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RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES INCLUDING BIOCHEMISTRY, COMMUNICABLE DISEASE AND IMMUNOLOGY, INTERNAL MEDICINE, NUCLEAR MEDICINE, PHYSIOLOGY, PSYCHIATRY, SURGERY, AND VETERINARY MEDICINE. VOLUME I

Walter Reed Army Institute of Research
Washington, D.C.

30 June 1973

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RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES, INCLUDING BIOCHEMISTRY, COMMUNICABLE DISEASES AND IMMUNOLOGY, INTERNAL MEDICINE, NUCLEAR MEDICINE, PHYSIOLOGY, PSYCHIATRY, SURGERY, AND VETERINARY MEDICINE

(Projects, tasks, and work units
are listed in Table of Contents)

Annual Progress Report
1 July 1972 - 30 June 1973

Volume I

Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington, D. C. 20012

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The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences - National Research Council.

SUMMARY

The various subjects covered in this report are listed in the Table of Contents. Abstracts of the individual investigations are included on the DD Form 1498 introducing each work unit report, and names of investigators are given at the beginning of each report.

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PROJECT 3A061101A91C
IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00
In-House Laboratory Independent Research

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6472	72 08 01	DD DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
72 07 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	100
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Hypersensitivity in the immunopathology of helminthic infections

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
70 11	72 08	DA	C. IN-House

17. CONTRACT/GRANT NA

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 72	3	70
FISCAL YEAR CURRENT 73	0.3	6

19. RESPONSIBLE DOD ORGANIZATION

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NAME: SADUN, E. H., Sc.D.

TELEPHONE: 202-576-3308

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: MOON, A. P. DA

NAME:

PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Radioactive; (U) Antigen; (U) Antibody; (U) Immunoglobulin; (U) Hypersensitivity; (U) Parasite

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) To determine the extent of hypersensitivity produced by helminthiases of military importance and their role in the immunopathology. This knowledge may be applied to developing effective agents for the prevention, or amelioration of these diseases in military personnel.

24 (U) After careful study of pertinent literature and consultations with immunochemists and pathologists, both classical and new methods will be used to obtain data from controlled experiments.

25 (U) Work unit is being combined with work unit 165, line item 3A061102B71Q.

*Available to contractors upon originator's approval

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1a

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA OB 6473	72 08 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
72 07 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	101
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)*

(U) Immunoprophylaxis of protozoan infections

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
70 11	72 08	DA	C. IN-House

17. CONTRACT/GRANT NA

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:*

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 72	3	70
FISCAL YEAR CURRENT 73	0.5	6

19. RESPONSIBLE DOD ORGANIZATION

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TELEPHONE: 202-576-3308

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: MOON, A. P. DA

NAME:

PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Immunize; (U) Irradiate; (U) Parasite; (U) Trypanosome

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) To develop means for inducing acquired immunity to protozoan infections of military importance. The results may be eventually applied to the prevention or suppression of these infections in military personnel.

24 (U) Conventional and experimental serological methods will be used to evaluate host reactions to infection following treatment with gamma irradiated parasite Data will be used to devise effective means of diagnosis, treatment, and/or immunoprophylaxis of infection.

25 (U) Work unit is being combined with work unit number 165, 3A061102B71Q.

*Available to contractors upon originator's approval.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6476	73 06 30	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
72 07 01	H. Termination	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	104
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede with Security Classification Code): (U) Management of Primary Hypertension and Autonomic Dysfunction Using Operant Conditioning Techniques

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 013400 Psychology 012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
70 11	73 06	DA	C. In-House

17. CONTRACT/GRANT: NA
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR — PRECEDING 72	02	50
FISCAL YEAR — CURRENT 73	1.5	40

19. RESPONSIBLE DOD ORGANIZATION
NAME: Walter Reed Army Institute of Research
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PII Redacted

21. GENERAL USE
Foreign Intelligence not Considered

20. PERFORMING ORGANIZATION
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TELEPHONE: 202-576-5522
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: Robinson, MAJ M.G.
NAME: Sodetz, CPT F.J. DA

22. KEYWORDS (Procede EACH with Security Classification Code): (U) Blood Pressure; (U) Operant Conditioning; (U) Human Volunteer; (U) Autonomic Dysfunction; (U) Psychosomatic Disease; (U) Primary Hypertension

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Procede text of each with Security Classification Code.)

23. (U) Development of behavioral techniques for the outpatient management of primary hypertension in military personnel through appropriate application of existing principles of operant and respondent conditioning. The systematic exploration of the role of these principles in the pathogenesis of hypertension, autonomic dysfunction, and psychosomatic disease under conditions of military performance related stress.

24. (U) Existing knowledge of operant principles is applied to both normal and hypertensive individuals to effect reductions in blood pressure of sufficient duration to warrant development and standardization of an optimal procedure for management of primary hypertension in outpatients. Techniques are extended to include the modification of esophageal, gastric, and colonic motility for the management of psychosomatically based gastrointestinal disorders. Concurrently, studies in non-human primates are conducted to update operant technology, to explore potentially productive methods for treatment of patients, and to facilitate development of required bioinstrumentation.

25. (U) 72 07 - 73 06 Tetrapolar impedance plethysmography was used to measure fluid volume and pulse volume in the whole leg over twenty-four (24) hours at bed rest. Findings establish a circadian and paradian variation in blood volume that is confirmed by pulse transit time measures. The utility of the impedance measurement technique was demonstrated, though electrode movement-produced artifacts remain a serious problem. Significant progress has been made in the study of blood pressure control in non-human primates using chronically implanted catheters. Effort in this work unit has been transferred to 91C OB6506 WU115 and 91C OB 6508 WU116. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 JUL 72 - 30 JUN 73.

*Available to contractors upon originator's approval.

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Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 104 Management of primary hypertension and autonomic dysfunction using operant conditioning techniques

Investigators.

Principal: Frederick W. Hegge, Ph.D.

Associate: MAJ Malcolm G. Robinson, MC; CPT John Varni, MSC

Description

A rapidly expanding body of literature (cf Barber, et.al., 1971) demonstrates the feasibility of applying operant conditioning technologies to the management of primary hypertension, autonomic dysfunction, and psychosomatic disease. The basic research strategy of this work unit involves: a) the development of appropriate non-invasive techniques for monitoring physiological variables of interest; b) the establishment of conditioning protocols that will effect changes in physiological functions of sufficient magnitude and precision to produce meaningful therapeutic and/or prophylactic affect; and c) the translation of laboratory procedures to techniques useful for clinical outpatient management. Specific areas of current research include: 1) the assessment of a commercially produced device specifically designed for operant control of blood pressure; and 2) the assessment of patterns of peripheral blood volume over twenty-four hours of bed rest.

Progress

1. A commercially available system designed specifically for training the voluntary control of systolic or diastolic blood pressure was acquired and evaluated. The system utilizes an automatically inflated and deflated pressure cuff that is equipped with a microphone for detecting Korotkoff sounds. Digital circuitry is employed to apply statistical criteria to the detection of coincidence between Korotkoff sounds and the QRS complex of the electrocardiogram waveform in order to establish systolic or diastolic pressure values. Circuitry is provided to track changes in either value and to provide visual and/or auditory feedback to subjects when pressure values increase or decrease appropriately.

The system was determined to be inappropriate for research use under this work unit for the following reasons: a) the repeated inflation cycles

conducted at the necessarily brief intertrial intervals would produce potentially harmful ischemia; b) the oft repeated inflation cycles would produce local vascular changes that would interfere with the achievement of accurate repeated measurements; and c) good agreement between the system's determination of diastolic pressure and that of trained clinical observers could not be achieved. On the basis of these findings, further experimental testing of the system was suspended.

2. The evaluation of a self-balancing tetrapolar bioimpedance measuring system in a study of the blood volume of the leg under bed rest conditions was accomplished. Five human volunteers each spent twenty-four hours in an isolation chamber. Measurements of oral temperature, pulse rate, systolic and diastolic blood pressure, thigh to ankle electrical impedance, and electrocardiogram were sampled at hourly intervals throughout the period. Subjects read and listened to music. A liquid diet was fed on demand and chamber illumination was under the subject's control.

Significant systematic temporal variations were observed in all of the measures taken. The systolic blood pressure changed by 10% between the high point at 2100 hours and the low point at 1500 hours. Diastolic pressure varied 17% between its peak at 2400 hours and trough at 1400 hours. As calculated from the electrical impedance of the leg and the change in impedance attributable to the passage of a blood pulse, the variation in blood volume per pulse was 24%, the most labile of the measurements taken. The peripheral propagation time for a pulse was calculated using the electrocardiogram record and the impedance pulse waveform. The propagation time varied 11% from fastest to slowest times at 2100 and 1400 hours respectively. The maximum and minimum propagation times were inversely related to limb impedance, a finding that supports the notion that peripheral electrical impedance is an excellent analogue of peripheral vascular resistance.

Though this study was undertaken to evaluate a piece of equipment, its findings have important implications for the study of the voluntary control of cardiac and autonomic functions. The basic biological rhythms described here have amplitudes that are greater than those ascribed to biofeedback training procedures. The relationship between biorhythmic lability and trainability of function has not yet been established. It is possible that definition of the timing and magnitude of endogenous autonomic fluctuations (in this case involving the cardiovascular system) will be critical for selecting patients who may benefit from operant conditioning techniques. It is already known that labile blood pressure is an

excellent prognostic indicator for the subsequent development of hypertension, and this index can almost certainly be refined through measurement of the time dimension of such lability. Since existing studies of biofeedback approaches to the control of hypertension have not included the circadian and ultradian fluctuations in basal or experimentally modified cardiovascular function, spurious estimates of the efficacy of such approaches cannot be ruled out. It seems possible that the temporal relationship between operant intervention and endogenous rhythmicity will be of major importance in maximizing the therapeutic utility of autonomic modification by conditioning techniques.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 104 Management of primary hypertension and autonomic dysfunction using operant conditioning techniques

Literature Cited.

References:

1. Barber, T., DiCara, L. V., Kamiya, J., Miller, N. E., Shapiro, D., and Stoyva, J.: Biofeedback and Self-Control. Chicago, Aldine-Atherton, 1971.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6513	73 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
	A. NEW	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMAR	61101A	3A061101A91C	00	105
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Antigenic Composition of Hepatitis B Antigen

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology 010100 Microbiology 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	CONT	DA	C. IN HOUSE

17. CONTRACT GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE d. AMOUNT:
e. KIND OF AWARD f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 73	1.5	45
	CURRENT 74	1.5	45

19. RESPONSIBLE DOD ORGANIZATION

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Divn of Communicable Dis & Immunol
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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: Bancroft, LTC William H.
NAME: Irwin, MAJ Gilbert R. DA

PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Hepatitis; (U) Virus; (U) Antigen; (U) Immunology

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) To define the antigenic composition of the several subtypes of Hepatitis B antigen, to investigate the biochemical basis for antigenic variation, and to relate antigenic characteristics to the immunologic response to infection and to immunologic protective mechanisms.

24 (U) Antigenic subtypes of hepatitis B antigen are purified and studied by several biochemical and immunologic methods; new methods for antigenic analysis are under development. The immune response of hepatitis patients is studied using new techniques and purified subtype antigens.

25 (U) 72 07 - 73 06. Isolation and purification of hepatitis B associated antigen (Australia antigen) from large volumes of serum was carried out by a commercial contractor in a manner similar to the analytical methods used at WRAIR (see 1971 and 1972 Annual Reports). Antisera to these purified antigens, free of human serum proteins, were prepared at WRAIR and used to identify and quantify the distribution of various antigenic determinants in patients with hepatitis around the world. A subtype designated as "ayr" from Taiwan was shown to be similar to but subtly distinct from adw. The problem of hepatitis B antigen subtypes is currently being dealt with by global distribution of reference reagents for standardization so that control of the disease from an immunological approach may be initiated. These reagents were also used to show that a new antigenic determinant reported by a civilian research laboratory was the same as the w determinant discovered by the WRAIR team. After comparing them by the present technology and with sera from many parts of the world, it was agreed to maintain the "w" designation published by the WRAIR laboratory. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1972 - 30 June 1973.

*Available to contractors upon originator's approval

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Project 3A061101A91C

Task 00 In-House Laboratory Independent Research

Work Unit 105, Antigenic Composition of Hepatitis B Antigen

Investigators.

Principal: COL P. K. Russell, MC; LTC W. H. Bancroft, MC
MAJ G. R. Irwin, MC; SFC M. Willhight
SP5 J. J. Karwacki; PFC R. H. Pinkerton

Description.

To define the antigenic composition of the several subtypes of hepatitis B antigen, to investigate the biochemical basis for antigenic variation, and to relate antigenic characteristics to the immunological response and immunologic protective mechanisms. Antigenic subtypes of hepatitis B antigen are purified and studied by several biochemical and immunologic methods; new methods for antigenic analysis are developed.

Progress

Characterization of HB Ag

1. Purification of HB Ag. A contract for the purification of HB Ag of specified subtypes was held by Electronucleonics Lab, Inc. (ELNI) of Bethesda, MD from 12 June 1972 to 30 May 1973. During that time, eight different human plasmas were processed, including ayw (2), adw (3) and adr (3) subtypes. Three of these were obtained from the SEATO Medical Research Laboratory (SMRL), the rest from WRAIR sources. The contractor met the requirements for antigen purification, concentration, packaging, labelling, and delivery in a satisfactory manner. With one exception, each antigen tested was found to be as free of human serum protein as most of the antigens previously prepared at WRAIR. The exception was a plasma that contained a lower concentration of HB Ag than the others. To produce a final product of sufficient antigen concentration, the contractor made a pool of several CsCl gradient fractions, some of which contained normal serum proteins. Table 1 lists the specifications of the antigens purified.

2. HB Ag subtyping reagents. Rabbit antisera were prepared to an assortment of antigens prepared by WRAIR or ELNI (Table 2). Most reacted with only one or two determinants. Very few showed distinct reactions with 3 determinants (Fig. 1). Representative subtyping reagents were bottled and lyophilized (Table 3). Subtyping reagents

Table 1 - Hepatitis B Antigens Prepared by Electronucleonics

Serum File No.	Plasma				Purified Antigen				
	Subtype	Vol	I.D. Titer	Particle Count/Log_{10}	Prep. No.	Cs Cl Fx	Vol. (ml)	Particle Count/Log_{10}	Particle Type
1. EC318	adw	405	1:2	11.82	143-80	10-14	27	13.46	20 nm
						15-17	22.5	13.15	"
2. EA047	ayw	400	1:8	12.63	143-83	10-14	50	13.36	"
3. DJ021	ayw	400		10.84	220-13	pool	17	12.54	"
4. ED003	adr	350	1:4	11.48	220-7	16	21	13.90	"
5. ED026	adw	350			220-58	13	10	13.36	"
						14	10	14.26	"
						15	10	14.41	"
						16	10	13.89	"
						17	10	14.23	"
6. ED010	adr	270			220-64	15	10	13.80	"
						16	10	14.00	"
						17	10	14.15	"
						18	10	14.04	"
						19	10	14.20	20 nm + fil.
7. EH016	adw	300		13.23	287-41	1 - 30 inclusive			
						10	10		10 - 15 nm
						12	10	12.54	20 nm
						14	10	12.62	"
						16	10	13.28	"
						18	10	12.54	"
						20	10	12.00	20 nm + fil.
						22	10	11.45	"
						23	10	12.50	20 nm, 40 nm + fil.
						24	10	10.00	
8. EH017	adr	300	1:4	12.48	287-44	1 - 30 inclusive			20 nm
				11.00		(10 ml each)			20 nm + fil.
				10.60					40 nm

Table 2 - HB Ag Antisera Prepared at WRAIR

Immunizing Antigen					Antiserum				
					HB Ab				
Subtype	Name	Serum No.	Volume	Rabbit No.	No. specificities	Subtype	Anti NSP	ID dil. for subtyping	Volume
ayw	Kane	DI 387	250	247					18
	Fuentes	DI 296	1	227	2	AY	±		11
				228	2	A(Y)	+		40
		DJ 020	8	238	2	(A)Y	+	1:2	51
		(DJ 019)	84	241	2	AY	±	1:4	34
				243	3 ?	AY(?)	±	1:3	27
				244	3	AYW	±		2(1/3)
	DeWitt	DJ 021	50	804		?		very weak at 1:2	55
	Engerbretson	EA 047	40	561	2	AY	+	1:2	33
				562	2	A(Y)	+	1:2	51
				563	2	A(Y)	+	1:2	20
				670	2	AY		1:2	
adw		EC 318	93	181	2	AD	+		22
			+50	182	3		+		13
				184	3	(A)D	+	1:3	45
				185		A+	+		19
				186	2	AD	+	1:6	30
		DZ 275	63	605	1	A	0	1:3	37
				607	3		+		15
		DZ 272	25	260					
				262					

Table 2 - HB Ag Antisera Prepared at WRAIR (Cont'd)

Immunizing Antigen				Antiserum					
					HB Ab				
Subtype	Name	Serum No.	Volume	Rabbit No.	No. Specificities	Subtype	Anti NSP	ID dil. for subtyping	Volume
adw (Cont'd)				264					
				821					
				822					
				825					
				826					
	Sheree Peters	ED 026	85	41	1	TR Reaction	a+	1:2	20
				806	0	NR			40
adr	SMRL 2794		0	256					15
	" "			257	3	ADR	0		3
	Begley	ED 003	4	558	2	AR	0	1:2	51
				559	2	AR	0	1:3	42
				560	2	AR			45
	Begley	DZ 278	3	603	2	AR	0		52
				604	3	ADR	0	1:4	20
				606	3	ADR	0	1:2-1:3	35
				627	2	(AD(R)	0	1:2	56
		ED 010	5 (Hedlund)	19					
				843	0	NR			50
				844	0	NR			42
				RYE					40

were distributed to several laboratories working with Hepatitis B (Table 4).

3. The identity of HB Ag determinants w and f. Dr. Scott Mazzur of the Institute of Cancer Research (ICR) reported the recognition of a new antigenic determinant of HB Ag with a human antiserum (Mazzur et al, 1972). She identified the new determinant as f and classified all antigens as either f+ or f-. Because of a similarity between the distribution of f+ and w+ sera, a collaborative study was conducted at the WRAIR with the specific object of clarifying the relationship between the w and f determinants of Australia antigen. For this purpose a panel of 28 sera containing Australia antigen was prepared at the ICR to represent diverse geographic areas and unusual combinations of determinants. The precipitin reaction of each serum was compared to that of standard antigens using unabsorbed antisera provided by each laboratory. The reference antigens were human sera E. H. and EC 318, considered to contain d+ f+ and adw subtypes,

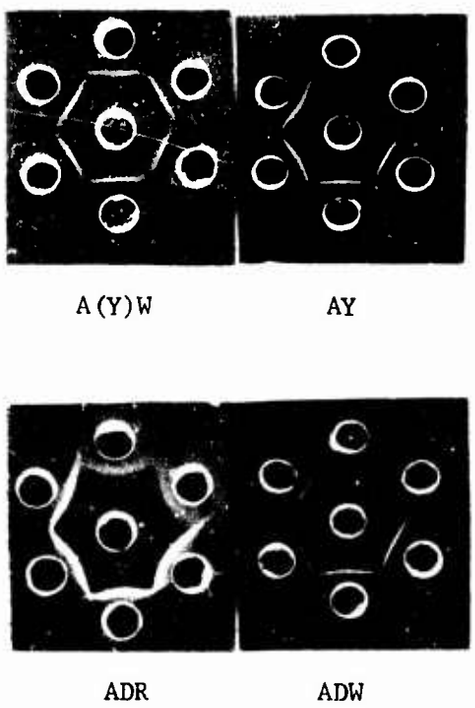


Figure 1. Representative immunodiffusion patterns of four rabbit antisera. HB Ag of 3 subtypes are arranged in the peripheral wells as indicated to the right. The rabbits illustrated are: A(Y)W, R244; AY, R563; ADR, R604; ADW, R607.

Table 3 - HB Ag Subtyping Reagents
Distributed June 1973

	Subtype	Serum no.	Optimum dil.	Source
Antigens:	ayw	EA 047	1:1	acute viral hepatitis (U.S.)
	adr	EH 017	1:1	Thai blood donor
	adw	EC 318	1:1	Thai carrier
Antisera:	AY	R 561	1:2	EA 047 (above)
	ADR	R 606	1:3	DZ 278 (U. S. carrier)
	ADW	R 182	1:4	EC 318 (above)

Table 4 - Hepatitis Investigators to Whom Subtyping Reagents Were Sent

1.	Dr. George L. Le Bouvier	Yale Univ.	New Haven, Conn.
2.	Dr. Sunitra Kamath	IMR	Kuala Lumpur, Malaysia
3.	Dr. Lars Magnius	Nat'l Bact. Lab	Stockholm, Sweden
4.	Dr. Ian Gust	Fairfield Hosp.	Victoria, Australia
5.	Dr. J. P. Soulier	CNTS	Paris, France
6.	Dr. Nakao Ishida	Tohoku Univ.	Sendai, Japan
7.	Dr. Joun Mateos	Cent Nat'l Viral & Ecol. San.	Madrid, Spain

respectively. Immunoprecipitin reactions of complete identity were observed between these two antigens when tested with four different antisera. For most of the comparative testing, two antisera were utilized. One was a human serum provided by Dr. M. Goldfield of the New Jersey State Department of Health and designated anti-af. The other was a rabbit anti-aw, prepared at WRAIR. Typical comparative reactions are shown in Fig. 2. The reactions produced by the two

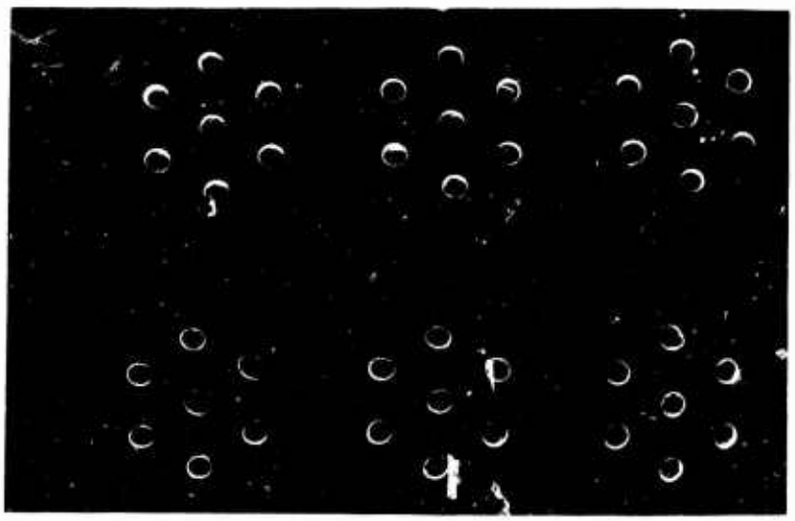


Figure 2. Immunodiffusion tests comparing precipitin reactions of 12 panel antigens with reference antigen E.H. (adf). The reference antigen is in the top and bottom wells of each pattern. Test sera are indicated by their panel number. The top slide has anti-aw in the center wells. The lower slide has anti-af in the center wells.

antisera did not differ significantly (Table 5). The one possible exception, serum 18, appeared identical to the reference antigen with anti-af but not with anti-aw. The spur seen with the latter antiserum was very weak. This antigen could be interpreted as being f+w-. However, when it was tested adjacent to f- antigens against anti-af, it failed to produce the spurs which would be expected if it were actually f+. It was concluded that this was an f-antigen and the apparent discrepancy between the two antisera was due to slightly suboptimal concentration for the formation of a weak spur. On the basis of these tests the f and w determinants appear to be identical. Since the w nomenclature has precedence over that of f, the determinant which is detected by the human serum and which was originally described as f, will be called w.

4. Presumptive recognition of subtype ayr. Dr. Paul Holland (NIH) provided a serum "Chu" for antigenic subtyping from a man from Taiwan. He had previously classified it as ayr, using monospecific absorbed antisera in an IEOP system (Holland et al, 1972). Because of a low concentration of antigen the serum was concentrated 2X and tested against ADR and ADW antisera by Rheophoresis (see 1972 annual report).

Table 5 - Comparison of the Precipitin Reactions of 28 Sera with Reference f+ and w+ antigens, Using Anti-af and Anti-aw Subtyping Antisera

Serum No.	Geographic Origin	Anti-af		Anti-aw	
		E.H.* adf	EC 318* adw	E.H. adf	EC 318 adw
1	South Africa	+**	+	+	+
2	" "	+	+	+	+
3	Bougainville	+	+	+	+
4	" "	weak reaction			
5	" "	-	-	-	-
6	" "	-	-	-	-
7	" "	-	-	-	-
8	" "	-	-	-	-
9	" "	-	-	-	-
10	" "	+	+	+	+
11	China	weak reaction			
12	U. S. A.	+	+	+	+
13	Japan	-	-	-	-
14	Peru	+	+	+	+
15	"	-	-	-	-
16	Bougainville	-	-	-	-
17	" "	-	-	-	-
18	" "	+	+	-	-
19	" "	-	-	-	-
20	" "	+	+	+	+
21	" "	+	+	+	+
22	" "	+	+	+	+
23	" "	-	-	-	-
24	" "	-	-	-	-
25	Malaita	-	-	-	-
26	U. S. A.	+	+	+	+
27	"	+	+	+	+
28	Africa	+	ND***	+	ND***

* Reference antigens

** (+) Reaction identical to that of reference antigen; (-) reference antigen formed a spur

*** Not done

The Chu antigen reacted like the adw antigen against the ADR antiserum but only the a determinant reacted with the ADW (Fig. 3). The reactions were faint but similar to those expected for an ayr. The presumptive classification of ayr could not be refuted. Another presumptive ayr, Ndorkas, was submitted by Dr. George Le Bouvier (Yale University). Although it reacted more strongly than Chu, the volume was insufficient for adequate testing. This serum came from New Guinea via Dr. Malcolm Simons of Singapore.



Figure 3. Immunodiffusion tests of serum Chu, containing HB Ag of presumptive ayr subtype. In each pattern the peripheral wells contain: top, ayw; right side, adw; bottom, adr; left side, Chu concentrated 2X. The center wells contain HB Ab, subtype ADR on the left and ADW on the right. The weak reactions of serum Chu are similar to those predicted for an ayr antigen.

5. Evidence for a fourth antigenic determinant. Electronucleonics provided six rabbit antisera to be tested. Each rabbit was immunized with the same purified adr antigen acquired from another source. Each antiserum was tested with 3 standard antigens representing 3 subtypes. A WRAIR ADR and ADW antiserum were included as controls. The results indicated a diversity of response by the rabbits (Table 6). The results reflected the different ways that similar animals may recognize and respond to the same foreign multideterminant antigen. No doubt it is this same individual reactivity that makes it difficult to consistently produce trivalent subtyping antiserum. One of the six animals produced antibodies that completely differentiated the ayw, and adw antigens yet still had 3 antibody specificities (Fig. 4). The crossing spur reaction was reproduced several times. The specificities could be removed by absorption with the standard antigens, but a thorough study of the absorbed antiserum to define the reactivity was not completed. The tentative

Rabbit HB Ab

ELNI 215

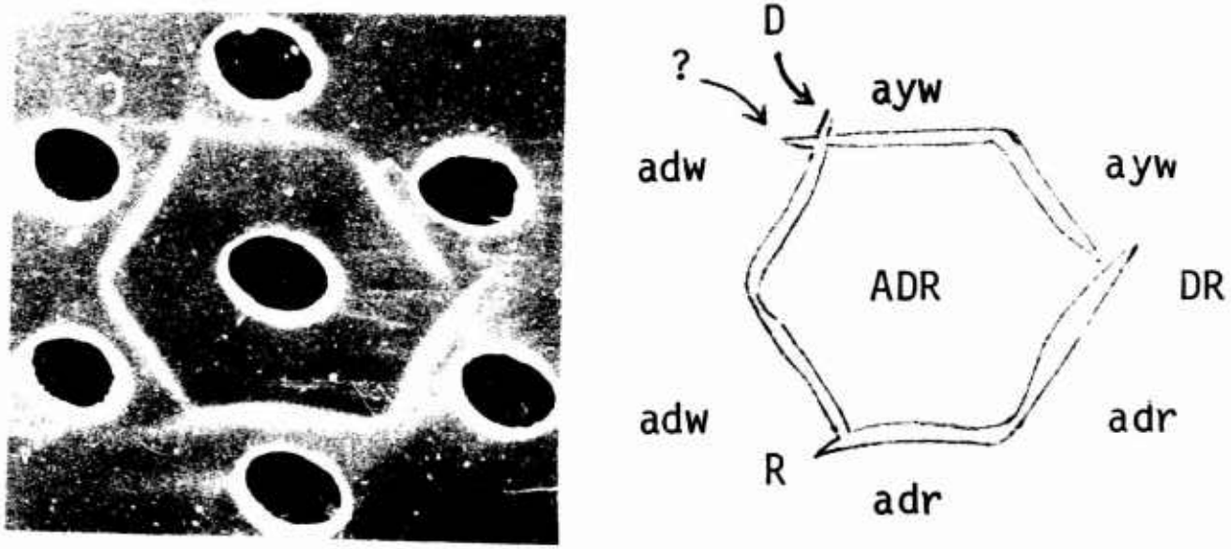


Figure 4. Immunodiffusion of ELNI rabbit 215 showing a reaction of nonidentity between the ayw and adw antigens. Although the rabbit was immunized with an adr antigen, no A antibody is apparent. A possible fourth determinant of the ayw antigen forms a new spur indicated by the question mark.

classification of this antiserum is DR4 with no A antibody. The 4 refers to some as yet un-named determinant on the ayw antigen.

Table 6 - Comparative Reactivity of Six Rabbit Antisera Produced from One HB Ag, Subtype adr

Source	Number	Reactivity
ELNI	ER 210	A
	ER 212	AD
	ER 214	AD
	ER 211	ADR
	ER 213	ADR
	ER 215	DR 4
WRAIR	R 559	ADR
	R 607	ADW

Summary

Further characterization of the antigenic determinants of hepatitis B antigen (HB Ag) was carried out. A bank of antisera was made by immunizing rabbits with purified antigens isolated from human plasma by WRAIR or a commercial contractor. The f determinant recently described by another laboratory was found to be the same as the w determinant previously described by this laboratory. However, there is evidence of a fourth antigenic determinant based on the double diffusion precipitation patterns in agar with an antiserum prepared against the commercially purified subtype adr. In addition, presumptive recognition of subtype ayr (previously not found but predicted) was confirmed.

527-825 O - 74 - 3

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DB OB 6477	73 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
72 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A061101A91C	00	107
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code) (U) Treatment of pain states arising from combat related peripheral nerve injuries by transcutaneous stimulation techniques (TST).

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012900 Physiology 016200 Stress Physiology 013400 Psychology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
70 07	CONT	DA	C. In-House

17. CONTRACT/GRANT: NA
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING FISCAL YEAR 73	1.5	25
CURRENT FISCAL YEAR 74	2.0	30

19. RESPONSIBLE DOD ORGANIZATION
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20. PERFORMING ORGANIZATION
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PII Redacted

21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Pain; (U) Peripheral Nerve Injury; (U) Psychophysiology; (U) Psychophysics; (U) Analgesics; (U) Human Volunteer (U) Combat

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Peripheral nerve injury, frequently resulting from a high velocity missile, may result in causalgia - a state of local hypersensitivity causing chronic pain. Transcutaneous stimulation techniques may relieve this pain and other forms of currently intractable pain. The validity of this analgesic method and the nature of its physiological mechanism are being identified.

24. (U) Using psychophysical and psychophysiological techniques, the amount of pain an individual experiences is measured before and after low level electrical transcutaneous stimulation. The individual's verbal reports of pain and his peripheral physiological reactions are both analyzed. Methods are designed to insure that the volunteer experiences the minimum possible amount of pain and discomfort.

25. (U) 72 07 - 73 06 A double blind study of TST in 21 normal volunteers was completed. High frequency DC pulses were effective in lowering pain judgements. Low frequency DC pulses were ineffective in lowering pain judgements relative to both control and high frequency stimulation conditions. Since this result was unanticipated, the study is being replicated. A study of sympathetic nervous system activity asymmetries during causalgia was developed and begun. Sympathetic activities in the four (4) limbs are compared pre- and post-treatment in causalgia, low back pain, and control patients. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 JUL 72 - 30 JUN 73.

*Available to contractors upon originator's approval

DD FORM 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 107 Treatment of pain states arising from combat related peripheral nerve injuries by transcutaneous stimulation technique (TST)

Investigators.

Principal: Frederick W. Hegge, Ph.D.

Associate: CPT J. R. Jennings, MSC; MAJ Albert Tahmoush, MC; Stanley Hall, M.A.

Description

The optimum clinical application of peripheral stimulation techniques for the treatment of pain depends on an understanding of the biological and psychological effects of the technique. Information on the reliability of results over time, efficacy in etiologically heterogeneous pain states, and specificity to pain sensation is important. Krasnegor, Jennings, and Fields (1972) found in a laboratory test on normal patients that TST resulted in a lowering of pain judgements and also a decrease in amplitude of skin conductance responses (an index of sympathetic activity). Meyer and Fields (1973) have used TST in the successful treatment of causalgia. Current research efforts have focussed on two major problems: (1) exact assessment of the effect of TST on normal volunteers, and (2) a study of causalgia with measurements of sympathetic activity prior to and following TST.

Progress

1. TST Effects on Pain Estimates and Skin Conductance in Normal Volunteers.

An extensive, double-blind study of TST in normal volunteers was conducted to clarify ambiguous results collected last year. The current experiment sought to 1) confirm the effectiveness of TST in comparison to placebo treatments; 2) delineate the unilateral or bilateral locus of pain decrement; and 3) demonstrate changes in sympathetic activity parallelling any change in pain judgements. After initial training in estimating the magnitude of induced pain, one group of volunteers was stimulated electrically in the median nerve distribution which was also the site of painful stimulus (third finger). A second group (peripheral group) was stimulated in an area unrelated to the site of the pain stimulus, while a third group (control) was not stimulated at all. Volunteers scaled pain bilaterally by alternating the hand stimulated from trial to trial. Pain was induced thermally, using a

modified Hardy-Wolff dolorimeter. Measures of skin conductance were collected concurrently, but have not been analyzed yet.

The results showed an unanticipated, but clear-cut reduction in pain judgements after the administration of high frequency TST on an area unrelated to the site of the painful stimulus. Low frequency TST in the median nerve distribution had no effect on pain judgement: pain judgements of the median and control groups were essentially equal. The pain reduction found for the peripheral group was confined to thermal stimuli considerably above the pain threshold level, i.e., threshold level pain was not influenced. The pain reductions were bilateral rather than unilateral. The results could not be attributed to changes in temperature, scaling biases, or awareness of the experimental manipulations. These findings are currently being replicated prior to the publication of such unexpected results.

2. Causalgia: A Study of Sympathetic Activity in Affected Patients.

Causalgia, a syndrome of localized severe persistent pain, may occur as the result of high velocity missile wounds sustained during active combat, and often leads to pronounced physical and psychological disability. The causalgic pain frequently can be relieved by interruption of sympathetic nervous system innervation to the affected area. The frequent success of this operation suggests that sympathetic nervous system may contribute to the causalgia syndrome. In order to examine this possibility, changes in pain severity are being correlated with sympathetic activity, as measured by skin conductance level (SCL) and blood volume pulse (BVP).

A twenty-four-hour study is being performed, during which the SCL of each of the four extremities and the BVP of the affected and homologous extremity is recorded. Three groups of subjects have been selected: (1) patients with causalgia; (2) patients with non-causalgic pain; and (3) normal volunteers. The patients with causalgia are studied on admission, following transcutaneous stimulation, and several days after interruption of sympathetic activity to the affected extremity. Each causalgia patient is matched to patients from the other two groups. These patients are studied for the same time intervals as the causalgia patient.

Preliminary studies in two cases of causalgia revealed a marked asymmetry between the skin conductance of the affected extremity and the remaining three extremities. The SCL of the unaffected extremities in patients with causalgia is within the range for the normal volunteers, whereas the SCL of the affected extremity is markedly increased. These results suggest that sympathetic activity to the affected area is increased in patients with causalgia. Development of a plethysmographic system for recording of skin

BVP will make possible the monitoring of two independent physiological variables which are both under sympathetic innervation. It is hoped that these studies will deepen our understanding of the physiological mechanism of pain and increase the tools available for its treatment.

Literature Cited.

References:

1. Krasnegor, N. A., Jennings, J. R., Orr, W. C., and Fields, H. L.: The after effects of peripheral nerve stimulation: Changes in psychophysical judgements and autonomic reactions during the scaling of cutaneous pain. Psychon. Sci. 26: 74-76, 1972.

2. Meyer, G. A., and Fields, H. L.: Causalgia treated by selected large fiber stimulation of peripheral nerve. Brain 95: 163-168, 1973.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6456	72 08 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
72 07 01	H. Term.	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	109
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Nuclear-Cytoplasmic Transplantation

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 08	72 08	DA	B. Contract

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: 71 08 EXPIRATION: 72 08
b. NUMBER: DADA-17-69-C-9182
c. TYPE: S.CT d. AMOUNT: 0
e. KIND OF AWARD: CONT f. CUM. AMT. 23,974

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 72	0.1	0
	CURRENT 73	0.1	0

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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ASSOCIATE INVESTIGATORS
NAME: Noyes, H. E. PhD
NAME: 1

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Nuclear Transplantation; (U)Enucleated Cytoplasm; (U)Cytochalasins; (U)Sendai Virus

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To extend preliminary observations on nuclear transplantation of mammalian cells and to apply the technique to immunologic studies of diseases of military importance.

24. (U) Through a general methodology which in preliminary evaluations has been shown to be feasible; consists of separation of nucleus and cytoplasm by cytochalasins, followed by fusion of nucleus and cytoplasm with Sendai virus.

25. (U) 72 07 - 72 08 It was determined that Cytochalasin B (CB) inhibited chemotaxis of polymorphonuclear leukocytes at doses of 1-2 mcg/ml but enhanced chemotaxis irreversibly at 0.1 mcg/ml. Further studies on transport of small molecules showed that CB is a competitive inhibition of glucose, glucoseamine, thymidine, uridine, and adenosine transport but not of choline transport. This contract terminated on 31 Jul 72, and a final report is expected.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6502	73 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
	A. New	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	0A061101A91C	00	112
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) SYSTEM STUDY OF MILITARY HEALTH CARE DELIVERY

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

(003500) Clinical Medicine 004200 (Computers) 016400 (Subsystems) 080400 (Admin&Mgmt)

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 03	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 73	.75	20
FISCAL YEAR CURRENT 74	3	75

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D.C. 20012

RESPONSIBLE INDIVIDUAL
NAME: Buescher, Edward L., COL, MC
TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Div of Biometrics & Med Info Proc
ADDRESS: Washington, D.C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: HANNEGAN, MICHAEL W., LTC, MC
TELEPHONE: 202-576-2011
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: WILBURN, JAMES, LTC, MSC
NAME: LEVY, JAMES, MAJ, MC DA

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Computers in Medicine (U) Health Care Delivery (U) Military Medicine

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23(U)To develop a systematic approach to implementation of computers to assist military health care providers to deliver care.

24(U)A multi-disciplinary group of physicians, nurses, pharmacists, dieticians, administrators, bioengineers, and computer specialists was formed to identify problem areas in information transfer in the delivery of health care in the military. This group then systematically addressed the solutions of the identified problems and is formulating a plan for implementation.

25. (U) 73 03 - 73 06 The multidisciplinary group met with other professionals to ascertain the requirements for military health care delivery. From this a systems study was initiated and is continuing to formulate implementation plans for solving the identifier problems.

*Available to contractors upon originator's approval.

DD FORM 1498 MAR 65

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 65 (FOR ARMY USE) ARE OBSOLETE

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6495	73 06 30	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
72 07 01	H. Term.	U	U	NA	NL	☐ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	113
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Effects of Physiological and Psychological Stress upon Infection and Diseases

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology 003500 Clinical Medicine 016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
64 10	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 72	2	65
FISCAL YEAR CURRENT 73	0	0

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL

NAME: Buescher, COL E. L.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of Neuropsychiatry

ADDRESS: Washington, DC 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Mason, J. W., MD

TELEPHONE: 202-576-3559

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Buescher, COL E. L.

NAME: DA

PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Respiratory Infection; (U)Stress; (U)Endocrine Response; (U)Hormones; (U)Viruses; (U)Sociology; (U)Personality Type; (U)Human Volunteer

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Definition and evaluation of various environmental and personal factors which contribute to physical and psychological stress experienced by military personnel, and determination of how these affect the overt clinical manifestations of naturally acquired infections. When factors are defined, efforts to modify clinical manifestations by modification of environment or human response to it are made.

24. (U) Endemic overt diseases in military populations were identified and studied for microbial etiology and variation in clinical manifestations. Environment in which they occur was defined. These findings were correlated with immunological susceptibility, physiological responses to environment and its stresses, and with personality types, and social backgrounds of patients. Factors suspected of influencing disease severity were evaluated in controlled experiments.

25. (U) 72 07 - 73 06 All hormonal and statistical analyses on this project are complete. Because of several striking pre-illness hormonal differences in the sick and control groups, it is felt that the findings of this study merit publication and two manuscripts have been completed in draft form, and a third manuscript is in preparation. One year absence of senior investigator coupled with departure from active duty in 1967 and 1968 of several of his colleagues has markedly delayed publication of these studies. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72-30 Jun 73.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 113 Effects of physiological and psychological stress upon infection and disease

Investigators.

Principal: John W. Mason, M.D.
Associate: COL Edward L. Buescher, MC

Description.

This study was designed to explore the possibility that stress-related, pre-illness changes in hormonal levels may play a contributory role in the pathogenesis of acute respiratory infections. The feasibility of the study was suggested by the high incidence of acute adenovirus infections in Army recruits during basic training in the winter months at Ft. Dix, New Jersey. Furthermore, the great majority of such illnesses usually are clustered during the third and fourth week of basic training. It was, therefore, possible to study a population in which a very high incidence of respiratory illness could be predicted within a designated two-week period.

Progress.

Two manuscripts of the findings in this study have been prepared, one dealing with the changes in thyroid hormones, the other with changes in corticosteroids and catecholamines, both before and during respiratory illness. A third manuscript is in preparation, dealing with an abnormal tendency for extremely high or extremely low adrenal, thyroid and gonadal hormone levels to occur during the pre-illness week in subjects destined to develop respiratory illness.

Summary and Conclusions.

Adrenal cortical, adrenal medullary, thyroid and gonadal hormonal abnormalities were noted during the pre-illness week in recruits who subsequently developed acute Adenovirus IV respiratory illnesses. The causes and possible pathogenetic significance of these pre-illness hormonal changes remain to be evaluated in future studies.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6504	73 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	8A. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
72 07 01	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	114
b. CONTRIBUTING				
c. ~~XXXXXXXXX~~				

11. TITLE (Procede with Security Classification Code)

Neurophysiological Control of Antibody Response

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002400 Bioengineering 016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 04	cont	DA	C. Inhouse

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER: NA

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 73	2	50
FISCAL YEAR CURRENT 74	2	50

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D.C. 20012

RESPONSIBLE INDIVIDUAL

NAME: Buescher, E.L., COL

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Division of Neuropsychiatry
Washington, D.C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Spector, N.H., Ph.D.

TELEPHONE: 202-576-3457

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Koob, G.F., CPT, MSC DA

NAME:

21. GENERAL USE

Foreign Intelligence not considered

22. KEYWORDS (Procede EACH with Security Classification Code) (U) Neurophysiology; (U) Neuropsychiatry; (U) Disease; (U) Trauma; (U) Stress

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Procede text of each with Security Classification Code.)

23. (U) The principal objectives of this research are (a) to study the role of the brain in the body's defenses against disease, and (b) to apply this knowledge to the prevention and cures of such diseases as malaria.

24. (U) Deficiencies in various parts of the brains of experimental animals were linked to the animals' ability to resist malarial infection and to recover from malaria.

25. (U) 72 07 - 73 06 A variety of lesions were placed in the brains of experimental animals. These animals and non-operated control groups were then infected with malaria. The development of the parasites in the blood as well as other changes such as body weight, temperature, and levels of circulating antibodies were measured daily or periodically in each animal. Studies of the brain tissues confirmed the exact locations of the lesions. Preliminary data analysis revealed that the position of the lesion not only affected the degree of proliferation of the malarial parasite, but also had an effect on the post-infection fever. Further data analysis is now in progress. These experiments will be extended with the object of gaining more information not only on brain influences upon disease, but also upon the origins and treatment of malaria and other diseases in man. These experiments are being conducted in collaboration with the Division of Communicable Diseases and Immunology at Walter Reed Army Institute of Research, and contribute to our knowledge of reactions of man to stress, wounds, and disease in battle and non-battle conditions. For technical report, see Walter Reed Army Instutute of Research Annual Progress Report, 1 Jul 72 - 30 Jun 73.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

PII Redacted

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 114 Neurophysiological control of antibody response

Investigators.
Principal: N. H. Spector, Ph.D.
Associate: LTC Carter L. Diggs, MC; CPT George F. Koob, MSC;
CPT Larry K. Martin, MSC.

DESCRIPTION.

This research was undertaken to study the role of diencephalic "centers," particularly in the hypothalamus, in the control and regulation of antibody production, the immune responses and other mechanisms in the body's defenses against disease.

The resources of the Division of Communicable Diseases and Immunology were utilized in several collaborative experiments.

PROGRESS.

Diencephalic influences upon the immune responses and resistance to disease:

Lesions in the anterior, or dorsomedial or posterior areas of the hypothalamus of rabbits, guinea pigs and rats have been shown to affect the immune response as measured by anaphylaxis and blood antibody titers (e.g. 1,2,3). This indicates that central nervous system induced alterations in the immune response might affect the body's resistance to a disease. Studies were pursued to determine the effects of discrete lesions in the hypothalamus upon the course of malaria infection.

Preliminary work included stereotaxic localization of the brain structures implicated, determination of proper lesion size, and a trial malaria infection. Anodal D.C. bilateral lesions were then placed in each of 24 male rats (Charles River, COBS Rat, CDF strain) (1): 8 in the anterior nucleus of the hypothalamus, 8 in the dorsomedial nucleus of the hypothalamus, and 8 in the posterior nucleus of the hypothalamus. Six "sham" animals had electrodes lowered into the hypothalamus; two at each location. All of these rats and 6 unoperated animals were injected with malaria parasites (Plasmodium berghei) six days postoperatively. Rectal temperature and body weight were recorded from all rats daily. Blood samples were taken for parasitemia measurements every other day, and blood was taken for antibody determinations at the beginning of infections, peak

of infection and end of infection (as determined by parasitemia counts, i.e. percent of parasitized erythrocyctes).

Histological analysis of the brains showed that approximately 75% of the rats had discrete bilateral lesions destroying a substantial part of the anterior, dorsomedial or posterior "nuclei." When the data were analyzed, according to confirmed lesions, there were no statistically significant changes in parasitemia levels in any of the operated groups; however, the unoperated control animals had the lowest parasitemia (statistically significantly lower than those of both the lesioned and sham-operated animals). The rats in all groups except the group with anterior lesions had a prolonged elevation in rectal temperature following the recovery from parasitemia. The blood samples for antibody measurements will be analyzed and a full interpretation of the above results must await these determinations. These experiments will be continued and extended.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 114 Neurophysiological control of antibody response

Investigators.
Principal: N. H. Spector, Ph.D.
Associate: LTC Carter L. Diggs, MC; CPT George F. Koob, MSC; CPT Larry K. Martin, MSC.

Literature Cited.

References:

1. Korneva, E.A. and Khai, L.M.: Effect of destruction of hypothalamic areas on immunogenesis. Fed. Proc. Trans. Suppl. 23(1): T-88-T92, 1964.

2. Macris, N.T., Schiavi, R.C., Camerino, M.S. and Stein, M.: Effect of hypothalamic lesions on immune processes in the guinea pig. Am. J. Physiol. 219(5):1205-1209, 1970.

3. Tyrey, L. and Nalbandov, A.V.: Influence of anterior hypothalamic lesions on circulating antibody titers in the rat. Am. J. Physiol. 222(1):179-185, 1972.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB6506	73 07 02	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	8a. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
	A. New	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A061101A91C	00	115
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Behavioral scheduling in psychosomatic disease

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002500 Clinical Medicine 013400 Psychology 012900 Physiology 016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	CONT	DA	C. In-House

17. CONTRACT/GRANT: NA

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 73	1.0	25
FISCAL YEAR CURRENT 74	1.5	40

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL

NAME: Buescher, COL E. L.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of Neuropsychiatry

ADDRESS: Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Sodetz, CPT F. J.

TELEPHONE: 202-576-2483

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Natelson, MAJ B. H.

NAME: Hegge, F. W., Ph.D. DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Operant Conditioning; (U) Psychosomatic Disease; (U) Autonomic Dysfunction; (U) Stress

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) This work unit is to systematically define functional relationships between behavioral variables, and the pathogenesis of psychosomatic disorders, e.g., hypertension and duodenal ulcer, and autonomic dysfunction, to identify potentially effective measures for prevention and treatment of this class of disorders.

24. (U) Empirically derived behavior principles are applied in studies of non-human primates to describe the functional relationship between behavioral variables such as, reinforcement, punishment, conditioned reinforcers, and conditioned aversive stimuli and activity, in a variety of physiological systems implicated in psychosomatic disorders. The focus is on arrangement of consequences for behavior, and the parallel control such consequences maintain over physiologic variables. Techniques include the development of effective means of chronically monitoring physiologic status of the organisms as well as the continuous updating of appropriate operant technology as a prerequisite to the exploration of potentially productive methods of treatment.

25. (U) 72 07 - 73 06 Food and water reward have been arranged for contingent increases in diastolic blood pressure to determine the effectiveness of reinforcement in establishing long-term changes in blood pressure. Pharmacologic agents modifying cardiovascular activity will be administered to determine whether reinforcement of blood pressure elevation produces any long-term modification of function relevant to the early etiology of essential hypertension. Non-human primates are being exposed to increments in the aversiveness of performance demands required of them. Each has been surgically prepared to permit direct observations of the gastric and duodenal wall and are being monitored for development of chronic peptic ulcer. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72-30 Jun 73.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A061101A91C IN-HOUSE LABOFATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 115 Behavioral scheduling in psychosomatic disease

Investigators.
Principal: CPT Frank J. Sodetz, MSC
Associate: MAJ Benjamin H. Natelson, MC;
Frederick W. Hegge, Ph.D.

Description.

The modification of autonomic function by perturbations in the environment has been well recognized for many years. However, only recently has the concept been extended to control by conditioned stimuli, that is, stimuli which acquire their controlling properties by virtue of repeated pairing with stimuli already effective in eliciting changes in autonomic activity. While appealing as a potential factor in the etiology of certain psychosomatic disorders, pathological responses to conditioned stimuli are known to be of limited duration occurring only in response to, and in the presence of, eliciting stimuli. It remained difficult to conceptualize long-term pathogenic changes in autonomic function resulting from brief exposures to such stimuli. In situations in which stressful stimuli were known to persist for extended periods, there was usually evidence of adaption to stress. However, within the past three years, evidence has begun to accumulate that the autonomic nervous system responds not only to eliciting stimuli antecedent to responses, but also to stimuli presented following changes in autonomic activity. It has been determined that autonomic activity can be brought under the control of environmental stimuli presented contingent upon a change in autonomic activity. This is precisely the arrangement of response and consequence that produces stable alterations in more conventional behaviors. To date, little has been done to systematically explore the control of autonomic function by manipulating the consequences of alterations in autonomic activity. The purpose of this work unit is to take advantage of these recent findings to examine the relationship of various arrangements of stimuli and responses to determine their role in the production and maintenance of the kind of chronic change in autonomic function thought to be significant in the etiology of psychosomatic disease and to develop models for the study of this class of disorders.

Progress.

Cardiovascular responses to acute behavioral stress and drug challenge following chronic elevation of arterial blood pressure maintained under behavioral control. A study has been undertaken in which four rhesus monkeys with arterial catheters are being run in a procedure requiring them to maintain a predetermined level of diastolic blood pressure in order to obtain food and water reward. The purpose of this study is to examine the response of the cardiovascular system to both acute behavioral stress (in this case, a 72-hour avoidance session), and to pharmacologic challenge by a number of drugs with specific actions on the cardiovascular system. Once the baseline response to stress and to pharmacologic challenge has been established, the animals will be required to maintain successively higher levels of diastolic pressure in order to obtain food and water reward. At the end of a 6-month period, during which elevated diastolic pressure will have been maintained for 12 hrs each day, these animals will again be challenged by both drugs and acute behavioral stress. By comparing the response to these challenges with responses obtained before blood pressure conditioning was instituted, it may be possible to begin to define some of the changes in cardiovascular function which take place early in the etiology of hypertension. To date, exposure to the initial 72-hr avoidance session has been completed and preparations are being made to introduce the pharmacologic challenge.

Analysis of the behavioral variables contributing to the development of gastro-intestinal ulcers. It has been demonstrated that exposure to behavioral stress can result in the development of gastro-intestinal disease in rhesus monkeys. To date, little work has been directed at identifying the specific behavioral variables that contribute to this finding Preparations were made to undertake a study designed to expose monkeys to behavioral stress in such a way as to permit identification of the procedural variables requisite to the production of gastro-intestinal pathology. The purpose of this study is to provide a useful model for examining the relationship of behavioral variables to psychosomatic disease. To date, only two monkeys have been run through a procedure that permits controlled specification of graded increments in the aversiveness of the situation in which the animal is required to perform. Both animals have been surgically prepared to permit direct gastroscopic observation of the stomach wall. To date, chronic ulcers have not been observed; however, acute lesions have been observed repeatedly.

527-725 O - 74 - 4

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION* DA OB 6508	2. DATE OF SUMMARY* 73 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SUM'RY	4. KIND OF SUMMARY A. New	5. SUMMARY SCTY* U	6. WORK SECURITY* U	7. REGRADING* NA	8a. DISP'N INSTR'N NL X	8b. SPECIFIC DATA - CONTRACTOR ACCESS ☒ YES ☐ NO

9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	6110 1A	3A0611 01A91C	00	116
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)*

Autocontrol of autonomic response

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

0 12000 Physiology 0 13400 Psychology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	CONT	DA	In-House

17. CONTRACT/GRANT NA

		18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
a. DATES/EFFECTIVE:	EXPIRATION:			
b. NUMBER:*		PRECEDING FISCAL YEAR 73	2	50
c. TYPE:	d. AMOUNT:	CURRENT FISCAL YEAR 74	2	60
e. KIND OF AWARD:	f. CUM. AMT.			

19. RESPONSIBLE DOD ORGANIZATION

NAME:* Walter Reed Army Institute of Research

ADDRESS:* Washington, D.C. 20012

RESPONSIBLE INDIVIDUAL

NAME: Buescher, COL E.L.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME:* Walter Reed Army Institute of Research
Division of Neuropsychiatry

ADDRESS:* Washington, D.C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME:* Hegge, F.W., Ph.D.

TELEPHONE: 202-576-5521

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

21. GENERAL USE

Foreign Intelligence not Considered

ASSOCIATE INVESTIGATORS

NAME: Robinson, MAJ M.G.

NAME: Redmond, MAJ D. DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Autonomic Dysfunction; (U) Psychosomatic Disease; (U) Human Volunteer; (U) Operant Conditioning; (U) Primary Hypertension

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Refinement of behavioral techniques for the outpatient management of primary hypertension and psychosomatic dysfunction in military personnel through extension of operant and respondent conditioning principles. The systematic investigation of the roles these principles play in the pathogenesis of autonomic dysfunction, primary hypertension, and psychosomatic disease under conditions of military performance-related stress.

24. (U) Knowledge of operant and respondent principles, in concert with simple and sophisticated non-invasive bioinstrumentation, are applied to normal and target individuals to effect clinically relevant changes in blood pressure, peripheral vascular resistance, and gastrointestinal motility. Procedures developed are extended, simplified, and standardized, where possible, to facilitate application on an outpatient basis.

25. (U) 72 07 - 73 06 Exploratory investigation conducted under DA OB 6476 WU 104 has demonstrated the unsuitability of pressure cuff monitoring techniques for blood pressure autocontrol. Impedance plethysmography techniques were explored and found to be useful under controlled laboratory conditions at their present stage of development. Future work will shift emphasis to the measurement and modification of peripheral vascular resistance using pulse arrival times, doppler flowmetry, and improved impedance plethysmography. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 JUL 72 - 30 JUN 73.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 116 Autocontrol of autonomic response

Investigators.

Principal: Frederick W. Hegge, Ph.D.

Associate: MAJ Malcolm G. Robinson, MC; CPT John Varni, MSC

Description

Preliminary work accomplished under DA OB 6476 WU104 provided requisite technical information for the development of a program of research dealing with the outpatient management of primary hypertension and psychosomatic dysfunction in military personnel through extension of operant and respondent conditioning principles. Emphasis is placed on the systematic investigation of the roles these principles play in the pathogenesis of autonomic dysfunction, primary hypertension, and psychosomatic disease under conditions of military performance-related stress.

Extensive knowledge of operant and respondent principles, in concert with simple and sophisticated non-invasive bioinstrumentation, is applied to normal and target individuals to effect clinically relevant changes in systolic and diastolic blood pressure, peripheral vascular resistance, and gastrointestinal smooth muscle activity. In addition, techniques for establishing perceptual discrimination of relevant internal states are explored. This step is necessary to bring internal function under the conscious and voluntary control of individuals so that their dependence upon electronic instrumentation for the achievement of control can be reduced and eventually eliminated. Procedures developed are extended, simplified and standardized to facilitate directed application by paramedical personnel on an outpatient basis.

Progress

1. A controlled environmental chamber and associated signal acquisition, modification, and control equipment have been designed and constructed. Significant advances have been made in the utilization of medium and large scale integrated circuits in the development of high quality, inexpensive amplifiers, filters, and feedback control devices designed to reduce dependence on expensive and complex laboratory equipment. Simplicity and reliability of operation are required for the establishment of a successful outpatient management program.

2. Work previously reported cited the extreme difficulty in obtaining reliable, non-invasive measures of systolic and diastolic blood pressure suitable for control applications. Not only do repeated inflation/deflation pressure cuff cycles cause discomfort to the subject, but also produce undesirable local vasular changes as well. Under these conditions, the close coupling between environmental and physiological events needed to establish functional control cannot be achieved. Consequently, attention was turned to techniques that would provide needed information on a heartbeat-to-heartbeat basis. Among these were tissue electrical impedance in a whole limb, peripheral propagation time of a blood pulse, and doppler ultrasonic flowmetry. Of these, the first two have received most attention to date.

Electrical impedance varies inversely with the fluid volume of the tissue segment across which measurements are taken. Since the major dynamic fluid constituent of tissue is blood, measurement of impedance parameters can provide useful information concerning the status of peripheral vasculature. Though promising, the technique has not been without its difficulties. The changes in impedance associated with the passage of a blood pulse have magnitudes in the order of one-tenth of one percent of basal tissue impedance. The equipment required to reliably detect such changes has, in the past, been complex, unstable, and difficult to operate. Recent advances in instrumentation have largely obviated these problems. Less tractable to ready solution has been the requirement for four electrodes that, in the case of whole leg measurements, completely encircle the limb. While the large area of such electrodes tends to minimize contact resistance problems, movement artifacts are a persistent problem and reliable measurements can only be taken when the limb is at rest. Future research will be directed to the application of flexible, epiconductive electrodes for impedance measurement.

In an attempt to verify the hemodynamic characteristics of impedance measurements and in search of simpler measurement techniques, blood pulse peripheral propagation time (PPT) was investigated. PPT is measured simply as the time between detection of the QRS complex of the electrocardiogram and detection of a blood pulse in the periphery. Since it is a temporal measurement, artifactual amplitude changes do not affect PPT measurements if the signal is of sufficient amplitude to be detected. In addition, there are many simple techniques for reliably detecting peripheral pulses, e.g., photoplethysmography. Although the information provided by PPT measurements is limited, their simplicity and reliability recommend them highly for vascular control applications if it can be shown that they reliably reflect blood pressure changes.

An investigation of the interrelationships between blood pressure, peripheral propagation time, and tissue impedance parameters was accomplished by reanalysis of data obtained during the study of five human volunteers undergoing twenty-four hours at bed rest (details are reported under DA OB 6476 WU1Ø4 in this volume). That study reported changes in the amplitudes of measures taken which varied up to 24% with time of day. This natural amplitude variation provided the opportunity to investigate the intercorrelation struction among six measured variables.

Significant negative correlations were observed between systolic blood pressure and PPT ($r=-.56$, $p<.01$), basal tissue impedance and PPT ($r=-.45$, $p<.05$), and the change in impedance attributable to a pulse and PPT ($r=-.39$, $p<.05$). Additional significant correlations were obtained between the calculated blood volume per pulse and diastolic pressure ($r=-.59$, $p<.001$), basal tissue impedance and diastolic pressure ($r=+.60$, $p<.01$), and the change in impedance attributable to a pulse and systolic pressure ($r=+.65$, $p<.001$). In view of these findings, the observation of no significant correlation between PPT and diastolic blood pressure is both surprising and unexplained at this writing.

The results of this natural experiment, while not definitive, do encourage the view that relatively simple, non-invasive measurements will provide adequate indices of cardiovascular activity that are suitable for the application of training technology. The required signals are readily obtained and of a character that will facilitate automatic processing by specialized control systems. Thus, encouraged, the research program is proceeding with the main line of inquiry, the voluntary control of cardiovascular parameters.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6512	73 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
	A. New	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	120
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Antigenic Components of the Cell Wall of Neisseria meningitidis

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	CONT	DA	C. In-House

17. CONTRACT/GRANT: NA
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e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
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FISCAL YEAR CURRENT 74	2	60

19. RESPONSIBLE DOD ORGANIZATION

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ASSOCIATE INVESTIGATORS
NAME:
NAME: DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) N. meningitidis; (U) Cell wall; (U) Protein; (U) Lipopolysaccharide; (U) Polysaccharide; (U) Antibodies; (U) Meningitis; (U) Human Volunteers

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) - To analyze the cell surface structure of the meningococcus with emphasis on extracting and purifying the dominant antigens. The goal is to develop candidate vaccines.

24 (U) - Protein, lipopolysaccharide and polysaccharide antigens will be characterized biophysically and immunochemically. Antibody responses of animals and humans will be measured by a variety of serologic methods. Antibodies will be purified by immunoabsorbent techniques.

25 (U) - 72 07 - 73 06 Protein, polysaccharide and lipopolysaccharide antigens of group B and C meningococci have been separated from the organisms by gentle extraction as "native" cell wall complexes. Individual antigens have been separated by Sephadex chromatography. Sera from immunized rabbits and infected humans have been shown to contain hemagglutinating antibodies directed towards protein and LPS antigens. Mice injected with isolated protein antigens were immune to challenge with intact meningococci. Bactericidal subtyping was performed on two strains of group B meningococci isolated from two separate illnesses in a child. By this method it was possible to show that the two strains were identical. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72-30 Jun 73.

*Available to contractors upon originator's approval.

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Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 120 Antigenic components of the cell wall of Neisseria meningitidis

Investigators.
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Associate: CPT Wendell D. Zollinger, MSC; MAJ J. McLeod Griffiss, MC; MSG Adam D. Druzd; SP5 Charles L. Pennington; Robert E.Mandrell; SP6 Dennis D. Broud.

1. Purification of meningococcal antigens.

Three major kinds of antigens have been demonstrated on the cell surface of the meningococcus:capsular polysaccharide, lipopolysaccharide and protein. Methods for isolating a native cell wall complex containing these three antigens and for separating the different types of antigen from the complex have been described(1). Several modifications of these procedures have been developed which allow purification of the protein and lipopolysaccharide antigens using milder more efficient methods.

Methods:

Native cell wall complex (outer membrane) was prepared as previously described(1) except that shear was applied to the organisms with an Omnimixer (Sorvall, Inc., Norwalk, CT) rather than by passage through a hypodermic needle. The isolated native complex was applied to a column of G-100 equilibrated with GED buffer (0.05 M glycine, 0.001 M EDTA, 0.5% DOC, pH 10). The sample was eluted with GED buffer at room temperature and the fractions assayed for protein, sialic acid and 2-keto, 3-deoxyoctonic acid.

Fractions containing the protein and those containing the lipopolysaccharide were pooled and the sample was recovered by one of two procedures: (1) The samples were extensively dialyzed against distilled water adjusted to pH 9.0 with a drop or two of ammonium hydroxide and then concentrated to one-tenth volume by ultrafiltration on a PM10 ultrafiltration membrane (Amicon Corp., Lexington, MA). This material still contained some bound deoxycholate which was removed by washing with 80 percent ethanol. One volume of the sample in 0.1 M NaCl was mixed with four volumes of cold absolute ethanol. The precipitate was pelleted, suspended in 95 percent ethanol, repelleted and then suspended in water. (2) Alternatively, the pH of the pooled fractions was lowered to 4.5 with HCl and the heavy precipitate consisting principally of deoxycholate was removed by centrifugation. The protein was essentially all precipitated along with the DOC but the LPS was only partially co-precipitated. The precipitates were washed at least three times with 10 or more volumes of absolute ethanol and the remaining precipitate

dissolved in water. The pH 4.5 supernatant from the LPS pool was concentrated to one-tenth volume on a PM-10 ultrafiltration membrane and the LPS was precipitated by addition of NaCl to 0.1 N and cold absolute ethanol to 80 percent v/v. The precipitate was collected by centrifugation, washed twice with ethanol and added to the LPS recovered from the pH 4.5 precipitate.

The LPS thus recovered contained a small amount of contaminating protein which was removed by extracting it with an equal volume of 90 percent phenol at 68-70°C as described by Westphal, et al[2]. The LPS was recovered from the aqueous phase by dialysis and lyophilization.

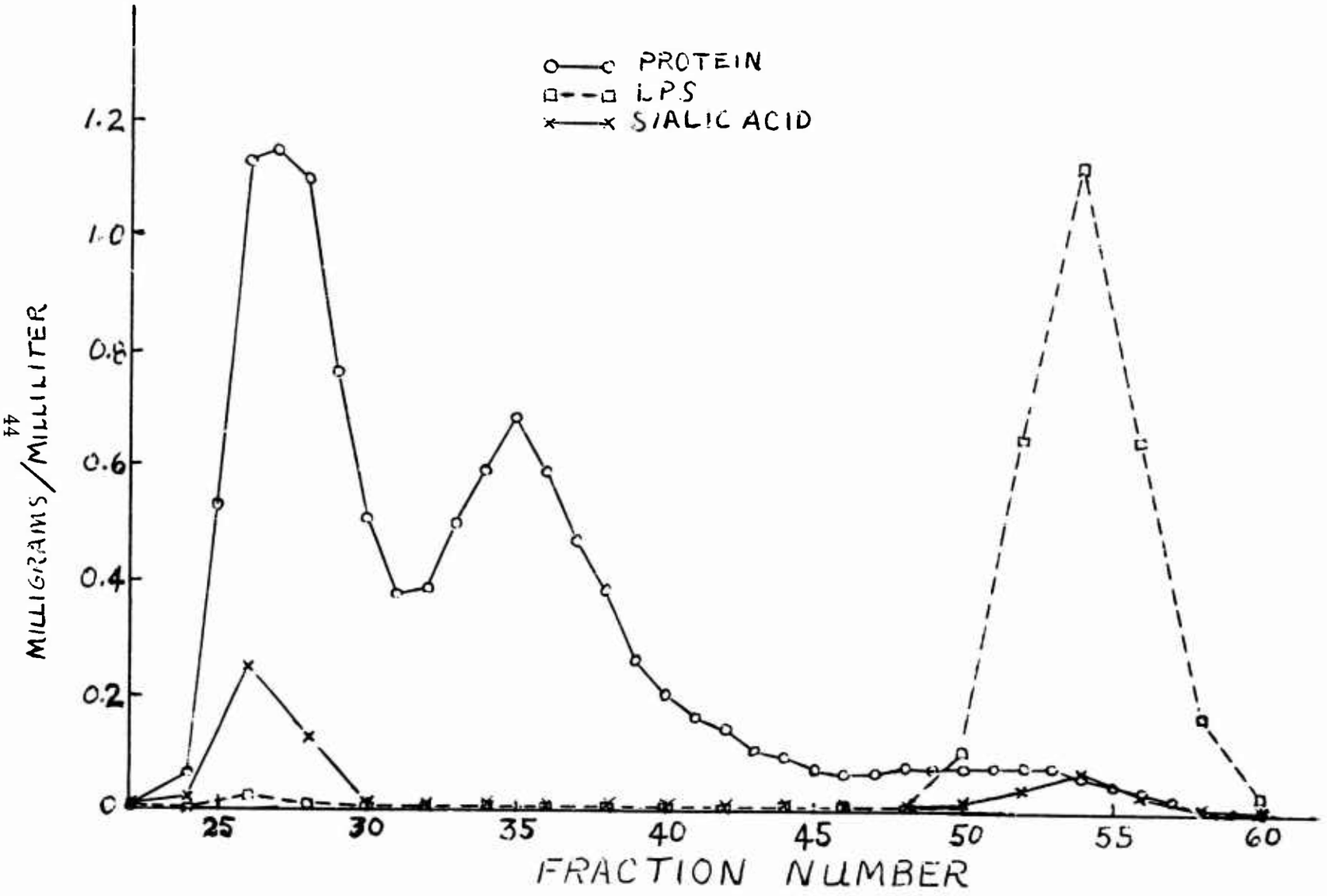
The protein recovered from the column contained only about 1-2 percent LPS and 1-2 percent nucleic acid, but contained 5-30 percent sialic acid depending on whether the cell wall complex was extracted from the organisms or recovered from the growth medium supernatant. Removal of the sialic acid (presumably present in the form of capsular polysaccharide) was accomplished by precipitating the protein with 25 percent trichloroacetic acid (TCA) at room temperature. The mixture was incubated at room temperature for one hour, centrifuged and the precipitate washed twice with distilled water.

Group B and C capsular polysaccharide was purified as described by Gotschlich et al[3] except that in the place of ultracentrifugation and multiple extractions with chloroform-butanol the partially purified product was chromatographed on Sephadex G-100 in GED buffer as described above for the cell wall complex. The polysaccharide, which eluted at the void volume, was recovered from the GED buffer as described above for the LPS fraction and subjected to cold phenol-water extraction to remove any remaining protein. This extraction was carried out as described by Westphal et al[2] except that the temperature was kept at 8-10°C rather than 68-70°C. All samples were dialyzed, lyophilized and stored under vacuum over P_2O_5.

Results:

Chromatography of native cell wall complex on Sephadex G-100 in GED buffer results in good separation of the protein from the LPS (Fig. 1). The protein elutes in one or two peaks depending on the strain from which the complex was isolated. The first peak which elutes at the void volume is the major peak and is consistently present while the second peak and its resolution from the first is strain dependent. The group specific polysaccharide elutes at the position of the first protein peak. The second protein peak may be recovered essentially free of LPS and group specific polysaccharide. Both protein peaks have been found to be immunogenic in rabbits (unpublished observations) and were normally combined in a single protein fraction unless otherwise indicated. Analysis of the two peaks by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) revealed that both peaks contain several polypeptide

Figure 1. Sephadex G-100 chromatography of native cell wall complex from 138I(C) organisms. The column (5x80 cm) was equilibrated and the sample eluted with buffer containing 0.05 M glycine, 0.001 M EDTA and 0.5 percent sodium deoxycholate pH 10.0. Thirty ml of sample was applied in the same buffer at pH 11.5. Flow rate was 60 ml/hr and fraction size was 18 ml.


PROTEIN
LPS
SIALIC ACID
MILLIGRAMS/MILLILITER
1.2
1.0
0.8
0.6
0.4
0.2
0
25
30
35
40
45
50
55
60
FRACTION NUMBER

chains and that one or more are common to both peaks.

Normally, a small amount of sialic acid was detected at a position exactly corresponding to the LPS peak. This could indicate the presence of one or more residues of sialic acid on the side chains of the LPS although contamination by group specific polysaccharide is an alternative explanation.

The extent of cross contamination of the purified antigens is indicated in Table 1.

Table 1. Composition of purified antigens

	Antigen		
Assay	Protein	LPS	Group specific polysaccharide
Protein	950**	9	5
KDO	1.08	70	ND
Hexose	4	250	6
Sialic acid	5.3	33	900
Nucleic acid*	20	5	10

* UV absorbance

** Values are expressed as micrograms per milligram dry weight and are the mean of determinations on 4-6 samples from different strains.

In general the contamination of any one antigen by either of the others is 1-2 percent or less. The actual contamination of LPS by group specific polysaccharide is unclear since part of the 3.3 percent sialic acid found to be present may be an integral part of the lipopolysaccharide structure. If meningococcal LPS, like E. coli and S. typhimurium LPS, contains three KDO residues per basic monomeric unit of 10,000-12,000 daltons then KDO would be expected to contribute 6-8 percent to the dry weight of the LPS. This agrees well with the observed value of 70 mcg KDO/mg LPS.

2. Indirect hemagglutination assay using purified antigens.

Methods:

Group specific polysaccharide:

IHA assays for anti-group specific polysaccharide antibody were carried out as described by Artenstein et al[4] except that sheep red blood cells (SRBC) were used with both rabbit and human sera and PBS buffer (0.02M Sodium Phosphate, 0.15M NaCl) at pH 7.2 was used with both group B and group C polysaccharides. Human sera were preabsorbed with an equal volume of 4 percent SRBC for one hr at room temperature to minimize nonspecific agglutination.

Lipopolysaccharide:

Purified LPS (2 mg/ml) to be used for sensitization of red cells was first treated with 0.1 N NaOH at 37°C for two hrs. Sufficient 0.5 N HCl was added to neutralize the solution and the antigen was then diluted to 1 mg/ml with PBS pH 7.2 and stored frozen. Washed SRBC at 4 percent were mixed with an equal volume of LPS at the optimal concentration (usually about 40 mcg/ml as determined by box titration against a standard antiserum) and incubated at 37°C for 30 min. After washing three times with PBS pH 7.2 the cells were diluted to 0.5 percent with pH 7.2 PBS containing 0.5 percent BSA. The test was carried out using the U-bottom microtiter plates as described by Artenstein[4]. The test was scored after two hrs at room temperature. Agglutination was graded 0 to 4+ and only those showing 3+ or greater agglutination were counted as positive. All sera used in IHA assays were inactivated by heating at 56°C for 30 min. In addition all human sera were preabsorbed with SRBC as described above.

Protein:

Lyophilized protein was suspended in water at 2 mg/ml and dissolved by dropwise addition of 1 N NaOH as necessary (usually to pH 11-11.5). The pH was then carefully lowered until the solution became slightly turbid (usually pH 9.5-10.0) or to pH 8.0 which ever occurred first. The solution was then diluted to 1.0 mg/ml with water and a small amount of sodium azide was added. The protein samples were stored in this state at 4°C until used. Just prior to use a sample of the protein was diluted with PBS pH 7.2 to the optimal concentration for sensitization (usually about 35 mcg/ml).

The SRBC to be used in the protein IHA assay were washed, tanned and sensitized following the method of Arbesman et al[5] with several modifications: a) Tanning and sensitization were both done at room temperature for 10 min. b) Following sensitization the cells were diluted with two volumes of 2 percent BSA in PBS pH 7.2, washed twice,

and suspended in the same buffer at a concentration of 0.75 percent. The test was scored after 4 hrs at room temperature or overnight at 4°C. Due to variability among different batches of SRBC in their tendency to auto-agglutinate when tanned and coated with the protein antigen, each batch had to be pretested using a standard antigen and antiserum. Known positive and negative sera were included in each test as controls. In addition, human sera were tested against unsensitized SRBC to detect nonspecific agglutination.

Results:

Specificity of HA tests; HAI tests:

In order to investigate the specificity of the polysaccharide, protein and LPS HA tests each of the tests was inhibited with serial dilutions of the homolcgous and heterologous antigens. The results as shown in Table 2 indicate that the tests are highly specific.

Table 2. Analysis of specificity of hemagglutination assays by hemagglutination inhibition.

	Antigen used to sensitize red cells		
Inhibiting antigen[1]	Group C polysaccharide	Protein fraction	Lipopolysaccharide
Group C polysaccharide	<0.9[2]	>1000	1000
Protein	>1000	1.8	1000
Lipopolysaccharide	>1000	>1000	1.8
Serum[3] dilution incubated with antigen	1:50	1:5000	1:20

[1] All antigens were purified from the group C strain 138I.

[2] Minimum concentration of antigen in mcg/ml required to inhibit agglutination.

[3] The same antiserum made against live 138I organisms was used in all assays.

No cross reaction of the group C polysaccharide and the LPS such as that described by Mergenhagen et al[6] was observed. Inhibition by the heterologous antigen required 500-1000 times more antigen than inhibition by the homologous antigen.

These results have been confirmed by studies using specific antibodies purified by sepharose-linked immunoabsorbent and by absorption

studies using antigens conjugated to Sepharose 4B. These studies are summarized in Table 3. Treatment of the protein-sepharose with pronase (or subtilisin) completely destroyed its ability to absorb out anti-protein antibody. Pronase (or subtilisin) had no effect on the Bsss-sepharose or the LPS-sepharose. This demonstrates that the antigenic determinants in the protein fraction are protein in nature while those in the LPS and Bsss preparations are not.

Activity of both IgM and IgG in the IHA tests:

Fractionation of immune rabbit and human antiserum on a G-200 Sephedex column was performed to determine the relative proportion of the total IHA titer resulting from IgM and from IgG. The results indicate that in both sera both IgM and IgG antibodies agglutinated sensitized red cells. In the rabbit antiserum IgG appeared to be responsible for most of the IHA titer while in the human serum (12 day convalescent serum from a patient with group B meningococcal disease) the IgM antibody appeared to produce more of the agglutination. Although these results give no quantitative information about the relative efficiency of IgM and IgG antibody in agglutinating red cells, they do indicate that the IHA tests are sensitive to both classes of antibody in both rabbit and human serum.

Human antibody responses to meningococcal protein and LPS antigens:

Human antibody response to the meningococcal group specific polysaccharides has been studied rather extensively. Additional studies have been published which have demonstrated human antibody response to nongroup specific antigens[7,8]. These studies, however, have not explicitly differentiated between protein antigens and LPS antigens. Using the three IHA assays specific for antiprotein, anti-LPS and anti-polysaccharide antibody we have compared human antibody responses to these three kinds of antigens.

Four groups were studied: 1) Individuals with group C systemic meningococcal disease. 2) Individuals with group B systemic meningococcal disease. 3) Healthy individuals who were free of the meningococcal carrier state at the time they received a group C polysaccharide vaccine, but subsequently became carriers of a group B meningococcus for at least two weeks prior to bleeding at six weeks postvaccination. 4) Healthy individuals who were not carriers at the time they received a group C polysaccharide vaccine and remained free of the meningococcal carrier state until they were bled at six weeks postvaccination.

Acute and seven day convalescent sera (groups 1 and 2 above) or pre- and six weeks postvaccination sera (groups 3 and 4 above) were assayed by IHA for antibodies against group B and group C polysaccharide and against protein and LPS antigens purified from five to seven strains (Table 4).

Table 3. Demonstration of specificity of antibodies directed against three major classes of meningococcal antigens.

Rabbit antiserum	Reciprocal of serum dilution versus indicated antigen used to sensitize cells: Group C polysaccharide	138I protein fract.	138I lipopoly-saccharide
Anti-138I (whole organism)	1024	32,768	128
Purified antibodies:			
Anti-138I protein	0	>4,096	0
Anti-138I LPS	0	0	32
	Group B polysaccharide	**99M protein fract.**	**138I lipopoly-saccharide**
Anti-99M (whole organism)	512	1,024	64
Absorbed with:			
Group B (Bsss) polysaccharide-sepharose	<4	1,024	64
Pronase treated Bsss-sepharose	<4	ND	ND
99M protein-sepharose	256	<4	32
Pronase treated 99M protein-sepharose	ND	1,024	ND
99 LPS-sepharose	256	1,024	<4
Pronase treated 99M LPS-sepharose	ND	ND	<4

Table 4. Human antibody responses to three different meningococcal antigens as determined by indirect hemagglutination.

Origin of serum	No. of individuals	Class of antigen used to sensitize erythrocytes			
		Group specific polysaccharide			
		C	B	Protein[b]	Lipopolysaccharide[c]
Meningitis cases					
Group C	8	5.5[a]	0.25	5.5	3.4
Group B	7	0.1	1.6	5.8	4.5
No illness[d]					
Non-carriers	10	6.0	N.D.	0.1	0.5
B-carriers	11	3.7	0.4	4.7	1.2

[a] Geometric mean increase in IHA titer expressed as the number of serial 2-fold dilutions. All sera were initially at a 1:4 dilution.

[b] Acute and 7 day convalescent (cases) or pre and 6 week postvaccination. Sera were tested against protein antigens from 5-7 different strains and the largest increase in titer for each pair of sera was used in computing the mean.

[c] Sera were tested against LPS from 7 different strains and the largest increase in titer obtained for each pair of sera was used in computing the mean.

[d] All subjects had received group C polysaccharide vaccine.

Protein and LPS antigens were purified from three strains which were isolated from the spinal fluid of three of the individuals with group B disease. These three preparations of protein and LPS were among the five to seven protein and LPS antigens against which all sera were tested. Thus, in all but three cases the sera were tested against protein and LPS antigens from heterologous strains. The protein and LPS antigens used were chosen to give a good representation of the most commonly encountered serotypes based on the bactericidal serotyping of Gold et al[9] and of Kasper et al[7]. Approximately half of the antigens were from group C strains and half from group B strains. Thus, in computing the mean increase in titer for a group of sera against the protein or LPS, the largest increase observed for each pair of sera against any of the set of five to seven antigens was used. Although caution must be used in making generalizations based on results obtained with a small number of individuals, the results in Table 4 do allow several interesting conclusions to be drawn:

(1) Humans who become ill with either group B or group C systemic meningococcal disease often respond with at least an 8-64 fold increase in antibody titer directed against both protein antigens and LPS antigens. All 15 cases had a 4-fold or greater increase in antibody titer against at least one of the protein antigens and one of the LPS antigens. In general, an anti-protein response was detected with six of the seven protein antigens used and an anti-LPS response with five of the seven LPS antigens used.

(2) This response to the protein and LPS antigens is fairly independent of the antibody response to the group specific polysaccharide. Although the individuals with group C disease showed a much higher rise to the group specific polysaccharide than individuals with group B disease, both groups responded to about the same extent to the protein and LPS antigens.

(3) Individuals who received the C polysaccharide vaccine and were not carriers during the period they were followed had a highly specific antibody response to the C polysaccharide.

(4) On the other hand, individuals who received the C polysaccharide vaccine and subsequently became B carriers for at least two weeks prior to the six week bleed, frequently responded with an increase in antibody directed not only against the C polysaccharide but also against protein antigens.

Eight of eleven carriers showed a 4-fold or greater rise in antibody titer to at least one of the protein antigens and of the three that did not respond, two had initial positive titers (1:32) to the protein. This group of B carriers showed only a marginal antibody response to the LPS antigens and virtually no response to the B polysaccharide. The poor antipolysaccharide response of group B carriers and cases has been previously documented by Brandt et al[10] using a

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radioactive antigen binding assay. The apparently poor anti-LPS response could be the result of not using homologous LPS antigens for coating the red cells.

The apparent difference in the response of the carriers and non-carriers to the C vaccine is not meaningful since only those non-carriers who had been shown to have a good antipolysaccharide response were chosen for study.

3. Immunogenicity of isolated protein antigens in animals.

The finding that humans respond to both group B systemic infection and group B nasopharyngeal carriage with a much higher rise in anti-protein antibody than in anti-B polysaccharide antibody suggests that the protein antigens are important and may be effective as a vaccine. Several studies on the immunogenicity of the protein fraction have been carried out with this in mind.

Protection in mice:

Two protein fractions from strain 138I(C) native cell wall complex (peaks I and II, Fig. 1) were tested for their ability to protect mice against lethal meningococcal infection. Peak I protein contained 5-10 percent group C polysaccharide while peak II protein contained no detectable group C polysaccharide. Neither contained more than 1-2 percent LPS.

Separate groups of 16 mice were immunized on day one with 138I(C) protein antigen at one of four serial 10-fold dilutions. On day 9 the mice were challenged with either 138I(C) or 99M(B) viable organisms. The results given in Table 5 indicate that both preparations of protein antigen were effective at relatively low levels in producing active protective immunity in mice. Protection against a group B organism with a group C protein antigen eliminates the possibility that contaminating C polysaccharide is responsible for this protection and suggests that the protein antigen can provide protection. It is not clear whether the lower ED_{50} obtained with peak I protein when the challenge was with the homologous strain is real and reflects additional protection resulting from antibody to the group C polysaccharide or whether it simply reflects the lower challenge dose given.

Acknowledgement: The mouse protection tests described above were carried out by Mr. Calvin Powell, Bioassay Section, Department of Biologics Research, WRAIR.

Antibody response in rabbits:

The protein-polysaccharide fractions (peak I plus peak II, Fig. 1) from three group C strains and two group B strains were tested for

Table 5. Protection in mice by protein fraction from meningococcal native cell wall complex[a].

Vaccine[b]	Challenge[c] Strain	No. of LD_{50}'s given[d]	ED_{50}[e] in micrograms
138I(C) Protein - Peak I	99M(B)	306	8.0
	138I(C)	38	<0.2
138I(C) Protein - Peak II	99M(B)	306	3.0
	138I(C)	38	0.9

[a]Assays were performed by Bioassay Section, Dept. of Biologics Research, WRAIR using 13 gm ICR mice.

[b]On day one 32 mice were injected at each of four serial 10-fold dilutions of antigen beginning at 200 µg/dose.

[c]On day nine half of the mice immunized at each antigen level were challenged with 99M(B) organisms and half with 138I(C) organisms.

[d]The LD_{50} for 99M(B) was approximately 15 organisms and for 138I(C) was approximately 13 organisms.

[e]Calculated by the Miller Tainter Probit method.

immunogenicity in rabbits. Two rabbits were injected with each preparation. Seven half-milliliter injections each containing 50 mcg of protein and 6-20 mcg of polysaccharide were given I.V. over a period of three weeks. The rabbits were bled at the end of the fourth week and the sera assayed for antibody against the protein, polysaccharide and LPS antigens. The results are given in Table 6. Since the titers of anti-LPS antibody are very low or zero it is likely that the bactericidal activity of the sera against organisms of the heterologous serogroup is due primarily to antiprotein antibody. Rabbits injected with protein-polysaccharide preparations from the group C organisms all responded with good titers of antibody to both the C polysaccharide and to the protein. Rabbits injected with similar preparations from group B organisms appeared to respond only to the protein antigens.

A group C polysaccharide-protein vaccine for human use would seem to have some advantages over the pure group C polysaccharide vaccine since it would possibly provide protection against some strains of groups A, B and Y, as well as against group C strains.

4. Serologic comparisons of meningococcal protein and LPS antigens.

Using a series of purified protein and LPS antigens from different group B and group C strains and antisera made against viable group B and group C meningococci, we have conducted some preliminary IHA studies to determine the extent of serological similarity or dissimilarity of the various protein and LPS antigens.

LPS antigens:

When a series of six antisera against different strains of viable meningococci were tested against each of the six homologous and heterologous LPS antigens, the result was a rather extensive degree of cross reactivity. Three of the sera, however, showed considerable specificity for their homologous LPS as compared to the other two. The results for these three sera are given in Table 7 and demonstrate that the three LPS antigens are all different. The observation that there is little or no cross reaction between the two group C LPS antigens further confirms the concept that the group specific polysaccharide is not identical with the side chains of the LPS but is rather a completely separate antigen.

Protein antigens:

A similar experiment to that described above with the LPS antigen was conducted using the protein IHA assay and a series of purified protein antigens. The results of this experiment (Table 8) also reveal rather extensive cross reaction among the protein antigens of this group of strains. Several of the sera, however, show considerable specificity (e.g. 418, 414, 415, 422). These strains have been shown

Table 6. Rabbit antibody response to protein-polysaccharide fraction isolated from meningococcal outer membrane.

Antisera[a]		Reciprocal indirect hemagglutination titers				Radioactive bactericidal assay	
Origin of protein-polysaccharide vaccine	Rabbit No.	Group specific polysaccharide		Lipopoly-saccharide	Protein	Reciprocal titer	Strain killed
		C	B	Homologous			
138I (C)	108	640	0[b]	0	320	640	99M (B)
	315	320	0	10	640	2560	99M (B)
126E (C)	114	320	0	0	640	40	6586 (B)
	116	320	0	10	320	160	6586 (B)
35E (C)	111	320	0	0	160 (20)[c]	160	121M (A)
	115	320	0	10	320 (10)	160	121M (A)
6586 (B)	105	20	0	20	320	640	118V (C)
	113	20	0	10	640	160	118V (C)
NE (B)	102	10	0	10	2560 (10)	160	118V (C)
	107	10	0	10	640	40	118V (C)

[a] Over a three week period each rabbit was given 7 half-milliliter injections each containing 50 mcg protein and 6-20 mcg polysaccharide. The rabbits were bled at the end of the 4th week.

[b] 0 = <1:10

[c] Reciprocal titer of prevaccination serum given in parenthesis. Where not given titers were less than 1:10.

Table 7. HA activity of rabbit antimeningococcal antibody versus lipopolysaccharide from homologous and heterologous strains.

Antiserum		Lipopolysaccharide antigen		
Immunizing strain	Rabbit No.	35E (C)	126E (C)	99M (B)
35E (C)	414	64[a]	<8	16
126E (C)	425	<8[b]	64	<8
99M (B)	422	8	16	64

[a] Reciprocal titer

[b] Serum dilution in first well was 1:8

Table 8. IHA activity of rabbit antimeningococcal antibody with protein antigens from homologous and heterologous strains.

Antisera[a]		Protein antigen on red cells					
Organism	Rabbit No.	60E	138I	126E	118V	35E	99M
C-60E	419	1 - 9[b]	0 - ≥9	0 - 2	0 - 5	0 - 5	0 - 8
C-138I	417	2 - 9	0 - ≥9	0 - 3	0 - 3	0 - 7	2 - 9
	418	4 - 10	0 - ≥9	0 - 0	0 - 1	0 - 5	4 - ≥11
C-126E	420	1 - 7	0 - 2	0 - 5	0 - 2	0 - 2	0 - 3
	425	0 - 7	0 - 1	0 - 5	0 - 2	0 - 2	0 - 1
C-118V	421	0 - 7	0 - 4	0 - 3	0 - 5	0 - 4	0 - 3
C-35E	414	0 - 6	0 - 0	0 - 0	0 - 0	0 - 9	0 - 3
	415	[illegible] - 8	0 - 1	0 - 1	0 - 3	1 - 7	1 - 5
B-99M	422	0 - ≥11	0 - ≥9	0 - 3	0 - 0	0 - 0	0 - ≥11
	423	0 - 10	0 - ≥9	0 - 6	0 - 1	0 - 3	1 - 11

[a] Antisera were made by injecting rabbits three times at two week intervals with viable meningococci of the strain indicated (approximately 10^8 organisms per injection). Rabbits were bled one week after the third injection.

[b] Indirect hemagglutination titer
Number of serial 2-fold dilutions: Pre-post.
Serum dilution in first well was 1:8 in all cases.

0 = <1:8

by bactericidal serotyping to each differ by at least one serotype antigen, but most of the organisms have more than one serotype antigen. An alternative explanation for the apparent specificity might be that individual rabbits have responded differently and that testing of more animals might result in a more uniform response.

Since most of the bactericidal serotyping sera were absorbed sera, similarly absorbed sera will have to be used in the protein IHA assay to determine if the same specificities can be demonstrated by this method as by the bactericidal reactions.

5. Bactericidal subtyping in recurrent meningococcal disease.

Techniques of bactericidal subtyping of meningococci which have been utilized to document differences between strains within a serogroup have previously been applied to problems of epidemiology[11] and antigenic analysis[10]. We have now had the opportunity to use this technique to study strains recovered from two separate illnesses in one child.

Case report:

An 18 month old female presented with a generalized seizure, fever, and maculopapular rash. Examination showed absence of meningitic signs, WBC 24,000/cu mm, CSF normal but blood culture yielded N. meningitidis group B. After seven days of intravenous ampicillin (100 mg/Kg/day) therapy the child was discharged. She remained well for two months at which time she developed vomiting, lethargy and neck pain. Fever and stiff neck were noted; WBC was 11,800/cu mm, a blood culture was negative, CSF was turbid, 420 WBC per cu mm (90 percent neutrophils), 35 erythrocytes, sugar was 44 mg% (blood sugar 160 mg%), gram negative diplococci were seen and N. meningitidis was isolated. Ampicillin was again administered for seven days with beneficial effects. Additional investigations revealed normal mastoid X-rays, negative test for glucose in nasal secretions, normal immunoglobulin levels for age (IgG = 680 mg%, IgA = 46. IgM = 44), negative throat cultures for meningococci in both parents.

Methods:

Strains from both illnesses were provided by Dr. Bannantyne of Canada. Serum from the first and tenth days of the second illness was available and required addition of penicillinase prior to performance of bactericidal tests. For these assays the radioactive bactericidal test (RBT) of Kasper[7] was used. Antipolysaccharide antibodies were measured by the radioactive antigen binding assay (RABA) of Brandt et al[10].

Results:

Strain #6939 from the blood culture of the first illness and #6940 from the CSF of the second illness were each identified as serogroup B by standard bacterial agglutination tests. In addition, both produced B polysaccharide as determined by inhibition of a polysaccharide hemagglutination assay. Both were inhibited by sulfonamide at a level of 0.4 mcg/ml. The patterns of bactericidal activity with specific serotyping antisera are shown in Table 9.

Table 9. Meningococcal serotyping based upon bactericidal patterns.

		Serotype antisera													
Group	Organism	1	2	3	6	8	9	10	11	12	13	15	16	23	24
C	60E	+	+	-	-	-	-	-	-	-	-	-	-		
C	138I	-	+	+	+	-	-	+	-	+	-	-	-	-	-
B	99M	-	+	+	+	+	-	+	-	+	+	-	-	-	-
B	6939	-	-	-	+	+	-	-	-	-	-	-	+	+	-
B	6940	-	-	-	+	+	-	-	-	-	-	-	+	+	-

+ = significant release of radioactivity

- = no cidal activity

Strains 6939 and 6940 gave identical reactions. Three other meningococcal strains are shown for comparison. Rabbit antiserum raised against 6939 induced antibodies to both organisms (Table 10). Absorption of this antiserum with each strain removed all or nearly all cidal activity against both, thereby confirming the identical nature of the two strains. Neither serum specimen from the patient (S-2714 and S-2715) showed anti-B polysaccharide antibodies by RABA. The initial serum of the second illness had a cidal titer of 1:40 against strain 6940, a level considered to represent immunity to systemic disease. There was an eight fold increase in titer to both strains in the 10th day serum.

Discussion:

By means of the bactericidal subtyping method it was possible to unequivocably demonstrate the identical nature of the strains from both illnesses. The presence of cidal antibodies in the earliest serum of the second illness is puzzling in view of the accepted pathogenetic mechanism for development of meningitis; namely, respiratory acquisition-mucosal invasion-septicemia-meningeal seeding. One might speculate, however, that this individual, whose initial episode was bacteremia, may have had a "violation" of the meninges during the trauma of a lumbar puncture (CSF was normal) which allowed entry of a few organisms. This

Table 10. Bactericidal antibody studies.

Antiserum	Titer vs indicated test organism 6939	6940
Rabbit		
6939 - Pre	<1:5	<1:5
6939 - Post	1:160	≥1:320
6939/6939*	<1:5	<1:5
6939/6940	1:10	<1:5
6939/6586	≥1:320	≥1:320
Patient**		
S-2714	1:10	1:40
S-2715	1:80	≥1:320

* Numerator = organism used to vaccinate rabbit
Denominator = organism used to absorb serum.

** Patient's paired sera were tested together but each organism was tested on separate days.

minimal focus of infection was held in check but not cured by antibiotics (? blood-brain barrier). After two months, and perhaps related to some unknown precipitating factor, organisms began to proliferate in the CSF which does not share in the humoral antibody system. Thus, the second episode was meningitis alone with negative blood culture.

The failure of this child to develop antipolysaccharide antibodies may not be abnormal. Although adults respond to group B disease with antibodies to the polysaccharide antigen the levels are low compared to group C infections. We have no experience with B polysaccharide antibodies in infants and children but at this age response to A and C polysaccharide vaccines is much less quantitatively than that of adults.

We have had specimens received this year from two Army recruits who have had two episodes of meningitis separated by a few months. In one case the first strain was serogroup Y; the second was group 135. The other patient had serogroup Y in both illnesses but both strains are not available for subtyping studies.

Summary and recommendations:

Protein, polysaccharide and lipopolysaccharide antigens of group B and C meningococci have been separated from the organisms by gentle extraction as "native" cell wall complexes. Individual antigens have been separated by Sephadex chromatography. Sera from immunized rabbits and infected humans have been shown to contain hemagglutinating antibodies directed towards protein and LPS antigens. Mice injected with isolated protein antigens were immune to challenge with intact meningococci. Bactericidal subtyping was performed on two strains of group B meningococci isolated from two separate illnesses in a child. By this method it was possible to show that the two strains were identical. Further exploration of the cell surface components of meningococci will be continued with the goal of developing candidate vaccines which have a broader reactivity than the highly specific capsular polysaccharides.

Literature Cited.

References:

1. Zollinger, W. D., Kasper, D. L., Veltri, B. J., and Artenstein, M. S. Isolation and characterization of a native cell wall complex from Neisseria meningitidis. Infection & Immun. 6:835-851, 1972.

2. Westphal, O., Luderitz, O., and Bister, F. Uber die extraktion von Bakterien mit Phenol/Wasser. Z. Naturforsch 7B:148-155, 1952.

3. Gotschlich, E. C., Liu, T. Y., and Artenstein, M. S. Human immunity to the meningococcus. III. Preparation and immunochemical properties of the group A, group B and group C meningococcal polysaccharides. J. Exp. Med. 129:1349-1365, 1969.

4. Artenstein, M. S., Brandt, B. L., Tramont, E. C., Branche, W. C., Jr., Fleet, H. D., and Cohen, R. L. Serological studies of meningococcal infections and polysaccharide vaccination. J. Infect. Dis. 124:277-288, 1971.

5. Arbesman, C. E., Rose, N. E., Kanton, S. Z., and Beede, R. B. Immunologic studies of ragweed-sensitized patients. J. Allergy 11: 317-332, 1960.

6. Mergenhagen, S. E., Martin, G. R., and Schiffmann, E. Studies on an endotoxin of a group C Neisseria meningitidis. J. Immunol. 90: 312-317, 1963.

7. Kasper, D. L., Winkelhake, J. L., Brandt, B. L., and Artenstein, M. S. Antigenic specificity of bactericidal antibodies in antisera to Neisseria meningitidis. J. Infect. Dis. 127:378-387, 1973.

8. Goldschneider, I., Gotschlich, E. C., and Artenstein, M. S. Human immunity to the meningococcus. II. Development of natural immunity. J. Exp. Med. 129:1327-1348, 1969.

9. Gold, R. and Wyle, F. A. New classification of Neisseria meningitidis by means of bactericidal reactions. Infection & Immun. 1:479-484, 1970.

10. Brandt, B. L., Wyle, F. A., and Artenstein, M. S. A radioactive antigen-binding assay for Neisseria meningitidis polysaccharide antibody. J. Immunol. 108:913-920, 1972.

11. Gold, R., Winkelhake, J. L., Mars, R. S., and Artenstein, M. S. Identification of an epidemic strain of group C Neisseria meningitidis by bactericidal serotyping. J. Infect. Dis. 124:593-597, 1971.

Publications:

1. Zollinger, W. D., Kasper, D. L., Veltri, B. J., and Artenstein, M. S. Isolation and characterization of a native cell wall complex from Neisseria meningitidis. Infect. & Immun. 6:835-851, 1972.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB6510	73 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY CTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
	A. New	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	122
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Radioimmunoassay Methodology for Drugs of Abuse

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	CONT	DA	C. In-House

17. CONTRACT GRANT NA

a. DATES/EFFECTIVE — EXPIRATION

b. NUMBER

c. TYPE — d. AMOUNT

e. KIND OF AWARD — f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 73	1	25
FISCAL YEAR CURRENT 74	2	50

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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TELEPHONE: 202-576-3600

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

ASSOCIATE INVESTIGATORS

NAME: Boone, B. J., Ph.D.

NAME: Emery, C. E. DA

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Radioimmunoassay; (U) Neuroendocrinology; (U) Morphine; (U) Antigen

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS. (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Objective is to develop a relatively specific and quantitative radioimmunoassay for the measurement of serum morphine concentrations which will be utilized in studies of drug addiction.

24. (U) A critical feature of the method development is the production of an effective morphine antigen by use of the haptene principle which has previously proven successful in the development of steroid radioimmunoassays.

25. (U) 72 07 - 73 06 A dihydromorphinone - (O-carboxy methyl oxime) - bovine serum albumen complex was synthesized as an antigen and, upon injection in rabbits, produced a suitably high titer of antibodies for immunoassay purposes. Using this antisera, a radioimmunoassay for serum morphine levels has been successfully developed, validated with regard to precision, recoverability and specificity. This method is currently being applied to the assay of samples from experimental studies. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72-30 Jun 73.

*Available to contributors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 68 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 122 Radioimmunoassay methodology for drugs of abuse

Investigators.

Principal: MAJ Scott E. Monroe, MC

Associate: Betty J. Boone, Ph.D.; Clarence E. Emery, B.S.

Description.

The purpose of this project is to develop sensitive, precise, and relatively specific radioimmunoassay procedures for the quantitation of heroin and similar drugs of abuse in both plasma and urine. These techniques, when fully validated, will be used to measure plasma and urinary concentrations of heroin and some of its metabolites in various endocrine and psychological studies concerned with drug addiction.

Progress.

1. Radioimmunoassay Development.

a. Production of antisera. Two different antigens were employed for immunization. The first antigen was synthesized by reacting dihydromorphinone with carboxymethoxylamine resulting in the formation of dihydromorphinone-6-oxime. This intermediary compound was then coupled to bovine serum albumin via carbodiimide. The second antigen, 3-carboxymethyl-morphine-rabbit serum albumin was obtained from a commercial source. Rabbits were immunized by multiple intracutaneous injections of antigen emulsified in complete Freund's adjuvant, each animal receiving a total dosage of 100 to 400 micrograms of antigen. Rabbits were boosted at approximately eight week intervals by the same route. A high percentage of rabbits immunized with either antigen produced antisera of suitable titer, specificity, and binding affinity for radioimmunoassay development.

b. Radioactive tracers. Dihydromorphine, tritiated at the 7, 8 positions, with a specific activity of approximately 50 curier per millimole, was purchased. Procedures for the production of iodine-125-morphine were also developed. In brief, iodine-125 was coupled to morphine through the use of chloramine-T The resulting product was purified on a column of Sephadex G-10.

2. Description of Radioimmunoassay.

a. Two distinct radioimmunoassay systems are functional. The first system (referred to as morphine-6-RIA) employs antibodies from the animals immunized with the morphinone-6-oxime derivative and tritiated dihydromorphine for the tracer. This system can detect and quantitate 20 picograms of heroin. In this system, heroin, morphine and probably 6-acetylmorphine have similar immunoreactivities. Morphine-3-monoglucuronide, however, is only about 1% as reactive as these other compounds. This property is especially useful in attempting to correlate immunoreactivity with biological activity since morphine glucuronide has very little, if any, biological activity.

b. The second radioimmunoassay system (referred to as morphine-3-RIA) employs antibodies from rabbits immunized with 3-carboxymethylmorphine and either tritiated dihydromorphine or morphine-I^{125}. In contrast to the morphine-6-RIA, heroin, morphine and morphine-3-glucuronide all have similar potencies in this system. This property allows one to more closely estimate the total concentration of narcotic in either urine or blood at any moment. The use of morphine-I^{125} is advantageous as this simplifies the overall procedure and reduces technician time. This system can also measure as little as 20 picograms of heroin. Further developmental studies are proceeding.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION* DA OB 6486	2. DATE OF SUMMARY* 73 07 01		REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SUM'RY 72 07 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY* U	6. WORK SECURITY* U	7. REGRADING* NA	8a. DISP'N INSTR'N NL	8b. SPECIFIC DATA-CONTRACTOR ACCESS ☒ YES ☐ NO	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	126
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)*

(U) Development of Capillary Blood Flow Sensors

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
71 09	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:*

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 73	2.25	56
	CURRENT 74	2.25	56

19. RESPONSIBLE DOD ORGANIZATION

NAME:* Walter Reed Army Institute of Research

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21. GENERAL USE

Foreign Intelligence Not Considered

20. PERFORMING ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Khouri, E. M.

NAME: Bass, B. G. DA

PII Redacted

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Blood Flow; (U) Shock; (U) Trauma; (U) Capillary; (U) Washout

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To develop equipment for the measurement, separately and simultaneously, of capillary blood flow during chronic experiments in various organs, e.g., liver, kidney, heart, skeletal muscle, in conscious unanesthetized animals.

24. (U) Beta radiation detectors encapsulated in special configurations are implanted surgically in trained animals and are used to measure the rate of washout of radioactive gasses, thus providing an estimate of capillary blood flow rate. Diodes which are at least 10 times more sensitive than those used in previous studies have recently become commercially available.

25. (U) 72 07 - 73 06 The incompatibility of avalanche semiconductor beta radiation detectors with the usual polymeric encapsulating materials has been overcome. The bare diodes are first passivated by coating with beeswax. Epoxy resin is then used to seal the diodes in a stainless steel case having a 1 mil front window. Four of six detectors so constructed are functional after three months, and are being evaluated and used in acute experiments. The two which have failed were implanted in chronic animal preparations. Tissue juices had penetrated the cable through the silastic insulating cover. Two more detectors are being constructed with polyvinyl chloride insulated cables for chronic animal testing. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72 - 30 Jun 73.

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527-825 O - 74 - 6

Project 3A061101A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 126, Development of capillary blood flow sensors

Investigators.
Principal: Ray A. Olsson, M.D., COL, MC
Associate: Edward M. Khouri; Billy G. Bass

Description.

Development and application of specially encapsulated beta radiation detectors for long term studies of capillary blood flow in conscious trained animals under the influence of physiological and pathological stresses.

Progress and Results.

The incompatibility of avalanche semiconductor beta radiation detectors with the usual polymeric encapsulating materials has been overcome. The bare diodes are first passivated by coating with beeswax. Epoxy resin is then used to seal the diodes in a stainless steel case having a 1 mil front window. Four of six detectors so constructed are functional after three months, and are being evaluated and used in acute experiments. The two which have failed were implanted in chronic animal preparations. Tissue juices had penetrated the cables through the silastic insulating cover. Two more detectors are being constructed with polyvinyl chloride insulated cables for chronic animal testing.

PII Redacted

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
	DA OB 6500	73 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
	A. New	U	U	NA	NL	☑ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	130
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)*

(U) Mechanisms of Action of Enteric Pathogens

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:*
c. TYPE d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 73	3	60
	CURRENT 74	2	50

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
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TELEPHONE: 202-576-2582
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: Rout, MAJ, W.R.
NAME: DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Diarrheal Disease; (U) Salmonellosis; (U) Shigellosis; (U) Enteric Disease; (U) Intestinal Absorption.

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Research efforts in this department are directed toward defining the pathophysiology of diarrheal disease, elucidating the effects of enteric pathogens on the gastrointestinal tract, and examining the influence of the intestinal flora on gastrointestinal function.

24. (U) Animal models are being used to evaluate the effect of diarrhea on intestinal salt and water transport, enzyme activities, and other functions.

25. (U) 72 07 - 73 06 Bacterial invasion and intramucosal multiplication are required for diarrhea production in Salmonella enteritis. A traditional enterotoxin is not involved. A useful in vitro tissue culture model has been developed to assess the invasive properties of salmonellae and the biochemical and metabolic effects of invasion. Diarrhea in the salmonella infected Rhesus monkey is a consequence of colonic as well as small intestinal disturbances in salt and water transport. Shigellosis in the Rhesus monkey is sometimes associated with diarrhea as well as dysentery. The diarrhea is a consequence of abnormal salt and water transport in the small bowel. In the Blind Loop Syndrome, small intestinal bacterial overgrowth, is associated with morphologic, biochemical, and physiologic small intestinal abnormalities. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 72 - 30 June 73.

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Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 130, Mechanisms of action of enteric pathogens

Investigators
Principal: Ralph A. Giannella, MAJ, MC
Associate: W. Robert Rout, MAJ, MC

Description.

The research activity in the Department of Gastroenterology this year has continued in the area of the pathophysiology of diarrheal disease and the mechanisms of action of enteric pathogens.

Progress and Results.

A paper reviewing the mechanism whereby shigellae causes enteritis and diarrhea has been published (1). Invasion of the mucosa and intramucosal multiplication of shigellae are essential to the development of enteritis and diarrhea.

A study dealing with the mechanisms of intestinal fluid loss by the salmonella infected rabbit ileum has been completed and published in abstract form (2).

A study dealing with the mechanisms whereby pathogenic strains of Salmonella typhimurium caused salmonella enteritis and induce fluid secretion and the pathologic consequences has been completed and published (3). This study revealed that only strains capable of invading the gastrointestinal mucosa can cause enteritis and induce fluid secretion. However, invasion of the mucosa and intraepithelial multiplication alone are not responsible for the fluid secretion. Another factor is involved, presumably a bacterial factor.

A paper reviewing the important modifying effect of gastric acidity on both enteric bacterial and parasitic enteric infections has been published (4). It describes that in the presence of reduced or absent gastric acidity, man appears to be more susceptible to salmonellosis, cholera, giardiasis and other enteric infections.

Studies dealing with the effects of overgrowth of the normal small intestinal bacterial flora on biochemical and physiologic events in the small bowel are in progress. Two abstracts have been published (5-6). A manuscript has been submitted for publication. These studies revealed that these intestinal bacteria damaged the intestinal epithelium causing a depression of various mucosal enzyme activities and impaired the transport of glucose and amino acids.

A study dealing with the morphologic effects of small intestinal bacterial overgrowth has been completed and an abstract published (7). A manuscript is in preparation. This study revealed that these bacteria caused morphologic damage to the brush border, mitochondria, and the endoplasmic reticulum.

Studies developing an in vitro model, He La cells, for assessing the invasive potential of strains of salmonellae have been completed and a manuscript is in press. This model correlates with ability of salmonellae to invade gastrointestinal mucosa in vivo and will be useful to study the effects of bacterial invasion on cellular biochemical and physiological processes.

An in vivo study, bacteriologic, pathologic, and physiologic, of the salmonella infected Rhesus monkey has been completed and an abstract published (8). A manuscript is in preparation. This study revealed that salmonella diarrhea is due to disturbances in salt and water transport in both the small and large intestine.

A study describing the mechanism of salt and water transport abnormalities in the salmonella infected rabbit ileum has been completed. An abstract has been published (9) and a manuscript submitted for publication. This study revealed that salmonellae, after they have invaded the ileal epithelium, stimulate the active secretion of salt and water.

A study describing the occurrence of folic acid deficiency occurring as a consequence of iron deficiency has been completed and two abstracts published (10-11). A manuscript is in preparation. Folic acid deficiency occurs not as a result of disturbances in intestinal function but as a result of ineffective erythropoiesis, intrameddulary hemolysis, and increased folic acid utilization.

Two letters to the editor dealing with the influence of gastric acidity on cholera and the occurrence of cholera-like diarrhea in salmonellosis have been published (12-13).

Studies dealing with intestinal transport in shigellosis in the Rhesus monkey is nearing completing. This study revealed that the small bowel secreted salt and water to contribute to the diarrhea of shigellosis. However, this process does not seem to be mediated by bacterial invasion of the mucosa or to mucosal morphologic abnormalities.

Examination of the effect of salmonellosis and shigellosis on the gastrointestinal bacterial flora revealed that the ileal and colonic flora are disturbed and remain so after the diarrhea has ceased. This study is nearing completion.

A study describing the effects of staphylococcal enterotoxin B on intestinal water and electrolyte transport revealed that staphylococcal enterotoxin inhibits sodium absorption and stimulated chloride secre-

tion. These effects are similar to those of cholera toxin. An abstract has been published (14).

Conclusions and Recommendations.

Work done in various animal models of infectious diarrhea, salmonellosis and shigellosis, indicate that both these groups of enteric pathogens cause enteritis and diarrhea by invading the gastrointestinal epithelium and multiplying within the mucosa. With salmonellae, mucosal invasion alone, does not seem to be responsible for fluid secretion and diarrhea but another bacterial factor, not a traditional enterotoxin, seems to be responsible.

Studies should be pursued, in both in vivo and in vitro model systems, to define what is the specific stimulus to bowel fluid secretions in these disorders, how it operates biochemically and physiologically, and how it can be modified. In addition, the relevance of other potential diarrheagenic mechanisms, i.e. hydroxylated fatty acids and unconjugated bile salts, must be assessed and their contribution to the diarrheas of salmonellosis and shigellosis defined.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 130, Mechanisms of action of enteric pathogens

Literature Cited.

Publications:

1. Formal, S. B., P. Gemski, R. A. Giannella, and S. Austin. Mechanisms of shigella pathogenesis. Amer. J. Clin. Nutr. 25: 1427-1432, 1972.

2. Giannella, R. A., S. B. Formal, G. Dammin. Pathogenesis of salmonella diarrhea. Clin. Res. 20: 880, 1972.

3. Giannella, R. A., S. B. Formal, G. J. Dammin, and H. Collins. Pathogenesis of salmonellosis: studies of fluid secretion, mucosal invasion, and morphologic reaction in the rabbit ileum. J. Clin. Invest. 52: 441-453, 1973.

4. Giannella, R. A., S. A. Broitman, and N. Zamcheck. The influence of gastric acid secretion on bacterial and parasitic infections of the intestine. a perspective. Ann. Intern. Med. 78: 271-276, 1973.

5. Giannella, R. A., P. P. Toskes, W. R. Rout. Reversible brush border injury and impaired sugar and amino acid uptake in the blind loop syndrome. Clin. Res. 21: 50, 1973.

6. Toskes, P. P., W. R. Rout, and R. A. Giannella. Antibiotic-reversible brush border damage in the blind loop syndrome. Clin. Res. 21: 527, 1973.

7. Giannella, R. A., P. P. Toskes, W. R. Rout, H. Jervis, and A. Takeuchi. Jejunal brush border and intracellular injury in the blind loop syndrome: a light, histochemical, and electron microscopic study. Gastroenterology 64: 733, 1973.

8. Rout, W. R., R. A. Giannella, S. B. Formal, and G. J. Dammin. The pathophysiology of salmonella diarrhea in the Rhesus monkey. Gastroenterology 64: 793, 1973.

9. Fromm, D., R. A. Giannella, S. B. Formal, R. Quijano, and H. Collins. Active ion transport across isolated ileum invaded by salmonella. Gastroenterology 64: 729, 1973.

10. Toskes, P. P., T. Bensinger, R. A. Giannella, and M. Conrad. Folic acid abnormalities in iron deficiency. Clin. Res. 21: 55, 1973.

11. Toskes, P. P., T. Bensinger, R. A. Giannella, and M. Conrad. Ineffective erythropoiesis with increased folate utilization secondary to iron deficiency. Clin. Res. 21: 569, 1973.

12. Giannella, R. A., S. A. Broitman, and N. Zamcheck. Gastric acidity and cholera. Ann. Intern. Med. 78: 780, 1973.

13. Giannella, R. A. Cholera-like diarrhea in salmonellosis. Lancet 1: 1185-1186, 1973.

14. Rout, W. R., L. R. Chang, and K. C. Huang. Effect of purified cholera enterotoxin and staphylococcal enterotoxin B on intestinal transport in flounder fish. Fed. Proc. 32: 1139, 1973.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	3. REPORT CONTROL SYMBOL
DA OB 6514	73 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
	A. New	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES:	PROGRAM E.EMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	167
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Spin Labeling of Biomolecules

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002300 Biochemistry 008300 Inorganic Chemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	Cont	DA	C. In-House

17. CONTRACT GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 73	3	75
FISCAL YEAR CURRENT 74	3	75

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Demaree, LTC G.E; Cella, CPT J.A.

NAME: Bass, B.G., MS; Copeland, E.S.,Ph.D.DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) ESR; (U) Spin label; (U) Spectrometry; (U) Drugs of Abuse

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) The technical objective of this work unit is to develop methods for analysis of important biochemicals and to study mechanisms by use of spin labeling and electron spin resonance (ESR) spectrometry.

24. (U) ESR and organic synthetic techniques will be used to develop reagents and methods for attachment of spin labels to biomolecules. Commercial reagents for spin labeling will be evaluated. Investigation of spin labels other than nitroxides will continue. Methods will be developed for synthesizing spin-labeled drugs and metabolites for use in studying pharmacokinetics of drugs of military importance, physico-chemical characteristics of receptor sites for drugs and enzymes, and binding site characteristics of antibodies. Spin label will be attached to antibiotic molecules to study pharmacokinetics of antibiotic action. Miniaturized high speed continuous flow analysis and antibody detection with application toward detection and analysis of microorganisms of medical interest and for clinical diagnosis will be evaluated.

25. (U) 72 07 - 73 06 Acquisition and installation of minicomputer for the Varian E9 ESR spectrometer was accomplished. The binding sites for spin-labeled morphine were located in rat brain synaptosomes. FRAT antibody to morphine was characterized. Stereoselective gels for detection of drugs of abuse were tested. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72 - 30 Jun 73.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

PII Redacted

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 167 Spin labeling of biomolecules

Investigators.
Principal: COL Charles R. Angel, MSC
Associate: SP4 Ronald L. Barber, B.S.; LTC Douglas J. Beach, MSC; MAJ Michael E. Boykin, MC; Edmund S. Copeland, Ph.D.; MAJ William E. Neeley, MC.

The objectives of this work unit are to develop methods for analysis of important biochemicals and to study mechanisms by use of spin labeling and electron spin resonance spectrometry and to apply these methods to clinical investigations where radioactive tags are not available, or are impractical or hazardous to use.

Work progressed in two areas:

1. The physico-chemical characteristics of drug receptor sites using spin label techniques.

2. Use of stereoselective gels in detection of drugs of abuse.

1. The physico-chemical characteristics of drug receptor sites using spin label techniques.

Using electron spin resonance (ESR) spectrometry in conjunction with spin labeled morphine (SLM) it is possible to estimate the binding of morphine to synaptosomes by determining the rotational correlation time (τ).

The rotational correlation of SLM added to synaptosome preparations is significantly inhibited. The binding is optimal after about 15 minutes at 23°C. Prolonged incubation at this or higher temperatures (up to 37°C) results in somewhat reduced SLM binding. Reversal of the order of incubation produced similar but less marked results. Preliminary investigations using very low SLM concentrations indicate that nearly 75% of the SLM present can be tightly bound by synaptosomes and can be released by incubation with nalorphine. These findings indicate that the interactions being studied occur at a specific site on the synaptosome which has some of the characteristics of a drug receptor. The rotational correlation time of spin labeled biomolecules offers a novel approach to the studies of biochemical mechanisms.

2. Use of stereoselective gels in detection of drugs of abuse.

Acidification of sodium silicate solution in the presence of levorphanol tartrate as a morphine template followed by purification and preparation of the resulting gel was found to produce silica gel with a marked binding affinity for normal and spin-labeled morphine. However, the association is of such a magnitude that once bound, the spin-labeled morphine cannot be released by addition of free morphine. Secondly, a satisfactory titration curve showing a break point in the strength of spin-labeled morphine by stereoselective gel could not be obtained. Apparently, the binding cannot be described by assuming a number of morphine specific binding sites/unit weight of gel. Lastly, no feasible simple way could be conceived in which the gel stereoselective for morphine could be readily adapted for rapid mass screening.

For these reasons, further development of stereoselective gels for use in detection of metabolites of drugs of abuse has been terminated.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 107 Spin labeling of biomolecules

Literature Cited.

Publications:

1. Copeland, E. S.: A simple method for estimating H_1 in ESR experiments - The microwave power saturation of gamma-irradiation induced glycylglycine radicals. Rev. Sci. Instr. 44: 437, 1973.

PII Redacted

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6518	73 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
	A. New	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	169
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)
(U) Membrane Transport in Gut

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	CONT	DA	C. In-House

17. CONTRACT/GRANT NA
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 73	1	25
FISCAL YEAR CURRENT 74	1	25

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

21. GENERAL USE
Foreign Intelligence Not Considered

ASSOCIATE INVESTIGATORS
NAME:
NAME: DA

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Stress Ulcer; (U) Diarrhea; (U) Stomach; (U) Membrane Transport

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) (a) To define mechanisms which govern permeability of the gastric mucosa in relation to stress ulcer and (b) to determine mechanism of diarrhea caused by an invasive organism, Salmonella typhimurium.

24 (U) (a) and (b) These problems have been approached using an in vitro system in which the spontaneous potential difference can be nullified and ion concentration gradients controlled thereby allowing distinction between active and passive transport processes.

25 (U) 72 07 - 73 06. (a) The primary mechanisms of action of salicylates and bile salt on causing increased back diffusion of hydrogen ion, other cations and anions by gastric mucosa have been defined. The primary effect of salicylates is to increase cation permeability only by increasing the negative field strength of the luminal border. Bile salt primarily acts as a detergent, increasing permeability to both cations and anions. Results also suggest that normal permeability is due to the prevailing field strength of the luminal border. (b) Ileal diarrhea of Salmonella enteritis is due to active secretion of Cl and inhibition of active Na transport. Invasion of the mucosa alone by Salmonella, however, is not essential for major alterations in spontaneous, active ion transport.

For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72-30 Jun 73.

*Available to contractors upon originator's approval

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Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-house laboratory independent research

Work Unit 169 Membrane transport in gut

Investigator.
Principal: LTC David Fromm, MC

An important conclusion of ileal diarrhea due to bacterial invasion has been established. Alterations in active rather than passive ion transport processes appear to account for a major portion of the diarrhea resulting from invasion of the ileum by Salmonella typhimurium. Active sodium and chloride absorption are inhibited and active chloride secretion is stimulated by the organism. However, luminal glucose stimulates active sodium absorption. This effect suggests a rational therapeutic approach for the symptomatic treatment of small intestinal diarrhea due to bacterial invasion. While the alterations in active ion transport are similar to those observed for cholera, the response of ileum invaded by S. typhimurium to theophylline differs from that observed for ileum exposed to cholera toxin. This suggests that alteration of a metabolic pathway other than cyclic AMP may also result in active chloride secretion.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-house laboratory independent research

Work Unit 169 Membrane transport in gut

Investigator.
Principal: LTC David Fromm, MC

Literature Cited.

Publications:

1. Fromm, D.: Na and Cl transport across isolated proximal small intestine of the rabbit. Amer. J. Physiol. 224: 110-116, 1973.

2. Fromm, D.: Intestinal absorption during hypovolemic shock. Ann. Surg. 177: 448-452, 1973.

3. Fromm, D.: Ileal resection, or disease, and the blind loop syndrome: current concepts of pathophysiology. Surgery 73: 639-648, 1973.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6532	73 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISS'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
72 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	170
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Biochemical Methodology and Laboratory Automation (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

008300 Inorganic Chemistry 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
66 10	Cont	DA	C. In-House

17. CONTRACT GRANT

a. DATES/EFFECTIVE: NA EXPIRATION

b. NUMBER

c. TYPE d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 73	6	150
FISCAL YEAR CURRENT 74	5	125

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER [redacted]

PII Redacted

21. GENERAL USE

oreign Intelligence Not Considered

ASSOCIATE INVESTIGATORS

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NAME: Bass, B.G., MS; Neeley, MAJ W.E. DA

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Automation; (U) ESR; (U) Toxicology; (U) Drugs of Abuse; (U) Gas Chromatography

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) The technical objective of this work unit is to develop and establish modern automated methods for qualitative and quantitative chemical analyses of military importance in medical laboratories.

24. (U) Automated analytical instruments, on-line digital computers and semi-automated manual techni[illegible] will be developed and adapted to identify and quantitate various compounds f[illegible]al and research uses. Emphasis will be placed on precise, accurate, a[illegible]nalytical systems, on simultaneous identification of a variety of compounds, [illegible]cal standardization and on practical systems for laboratory and intensive c[illegible]ities use. Efforts will be focused on fast identificati[illegible]nd quantitation [illegible]rugs of abuse and their principal metabolites, the utiliz[illegible] of simplified automated analytical systems for data collection and on standar[illegible] of techniques and equipment for automated laboratory systems.

25. (U) 72 07 - 73 06 Analytical chemistry methodology development [illegible]d appl[illegible]on was continued in this work unit. Implementation of multiple gas chromatographs controlled by a central automated processor was accomplished. Evaluation of an automated microanalytical continuous flow analysis system was completed. Use of small, inexpensive programmable electronic calculators as central processors for semi-automated methods such as ESR, FRAT, and RIA was initiated. Use of mass spectrometry in detection and identification of drugs of abuse was expanded. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72 - 30 Jun 73.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 170 Biochemical methodology and laboratory automation

Investigators.
Principal: COL Charles R. Angel, MSC
Associate: SP4 Ronald L. Barber, B.S.; Billy G. Bass, M.S.; LTC Douglas J. Beach, MSC, Betty J. Boone, Ph.D.; Nesbitt D. Brown, B.S.; CPT James A. Cella, MSC; Larissa deBaare, M.D.; LTC Gale E. Demaree, MSC; James E. Doolittle; PVT Aubrey L. Kiser, B.A.; Robert T. Lofberg, Ph.D.; PVT Burton L. Markham, B.S.; Edward J. Matusik, B.S.; MAJ William E. Neeley, MC; Dominic F. O'Donnell; Helen C. Sing, M.S.

The objectives of this work unit are to develop and exploit new laboratory methods for application to automation in order to provide rapid, precise and accurate qualitative and quantitative chemical analyses in support of military medicine.

During this report period, progress was made in the following areas:

1. High pressure liquid chromatography.
2. Automated miniaturized continuous flow systems.
3. Portable fluorometric systems for field application.
4. Organic synthesis.
5. GEMSAEC.

1. <u>High pressure liquid chromatography</u>.

The advent of the application of high pressure pumping systems to small bore liquid chromatographic columns generates a novel system that is capable of highly sensitive, high resolution separation and detection of small quantities of material from small samples. The use of this system can separate and detect morphine, monoacetylmorphine, diacetylmorphine, codeine, and morphine-3-glucuronide from aqueous solutions. This method is being evaluated to test the feasibility of its modification and subsequent application to a range of biological materials.

527-825 O 74 - 7

2. Automated miniaturized continuous flow systems.

An automated microanalytical system for detection and quantitation of drugs of abuse in biological materials using the FRAT has been designed and a prototype has been fabricated. Basically, the system employs a miniaturized continuous flow proportioning pump, specially designed quartz flow through cavity inserts for the ESR spectrometer and a specially designed electronic bubble gate. The ESR recorder output is converted to digital output which is interfaced with a programmable calculator for calculations and process control. Prototype evaluation reveals that the overall quantitative coefficient of variation is less than 3%. The system, exclusive of the ESR spectrometer, has been evaluated for applications to other microanalytical methods using visible and UV spectrophotometers. These studies reveal extensive potential applications for this system.

3. Portable fluorometric systems for field application.

NASA sponsored the development of a fiber-optics, ratio fluorometer using sharp-cut filters as the detection device for the pseudomorphine fluorophore. This unit is being evaluated for field use. Liquid-liquid extraction of morphine was compared for water and quality control urine samples. The data indicate that partitioning between water at pH 9.5 and chloroform-n-butanol (10% volume/volume) is approximately 96% in the direction of the organic phase. With urine, the minimum concentration necessary for detection using this instrument is 1.0 µg/ml.

4. Organic synthesis.

Derivatives of drugs which can be conjugated to protein to produce antigenic haptens in order to obtain specific antisera for immunoassays were synthesized.

Dihydromorphine-6-carboxymethoxyamine was synthesized from dihydromorphine and carboxymethoxyamine. Methadone-6-carboxymethoxyamine was similarly prepared from methadone. 6-O-carboxymethylmorphine was prepared from morphine and chloroacetate. Morphine-6-hemisuccinate was synthesized from morphine and succinic anhydride. The bovine serum albumin conjugates of these compounds proved to be antigenic in the rabbit.

Stable nitroxide free radicals to be attached to biomolecules for study by ESR spectrometry were synthesized. The syntheses of deuterated 2,2,6,6-tetramethyl-4-oxypiperidine-1-oxyl and 3,4-dihydromorphine-6-oxazolidine-N-nitroxide are in progress.

Spin-labeled morphine was synthesized from morphine base and 3-(2-iodoacetamido)-2,2,5,5-tetramethyl-1-pyroldinyloxyl.

5. GEMSAEC.

The GEMSAEC has been employed for analysis involving enzyme kinetics. Existing enzyme systems are being studied to assess the effects of drugs of abuse upon these systems. Thus far, the UDPG test has been evaluated. There is no evidence that any commonly abused drugs have any effect on this test.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6440	73 06 30	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
72 07 01	H. Term.	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	181
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Development of a Meningococcal Immunizing Agent

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
67 09	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 72	3	65
FISCAL YEAR CURRENT 73	2	50

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: DA

NAME:

PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) N. meningitidis; (U) Meningitis; (U) Polysaccharides; (U) Vaccines; (U) Proteins; (U) Human Volunteers

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To isolate, purify and characterize antigens from meningococci. To determine the protective capacity of immunogenic fractions. The goal is to develop vaccines against each of the antigenic types of meningococcus producing disease in military populations.

24. (U) To survey military and selected civilian populations to determine the prevalence of those serogroups of N. meningitidis causing disease. Polysaccharides and proteins will be purified and characterized by chemical and physicochemical methods. The response of animals and human volunteers will be measured by hemagglutination and bacterial antibody tests.

25. (U) 72 07 - 73 06 Group C meningococcal disease has essentially disappeared from recruit training centers and total number of meningococcal cases has been only 32. Much of this reduction is undoubtedly related to routine use of group C polysaccharide vaccine. Correlation of antibody responses after group C vaccines with molecular size by Sepharose 4B chromatography showed no difference in potency with MW varying from 520,000 to 2.2 million. Group A vaccines lost considerable immunogenicity after 2 years storage. A group B meningococcal cell wall protein was immunogenic in rabbits, but poorly so, if at all, in human volunteers. This work unit was consolidated with work unit 168, Bacterial Diseases, Project 3A061102B71Q, Communicable Diseases and Immunology. Future progress will be reported therein. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72-30 Jun 73.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 181 Development of a meningococcal immunizing agent

Investigators.
Principal: Malcolm S. Artenstein, M.D.
Associate: Brenda L. Brandt; CPT Wendell D. Zollinger, MSC; MAJ J. McLeod Griffiss, MC: MSG Adam D. Druzd; SP5 Charles L. Pennington; SP6 Dennis D. Broud; Robert E. Mandrell; Martha D. Tingley; Herman Schneider, Ph.D.

1. Epidemiology of meningococcal meningitis.

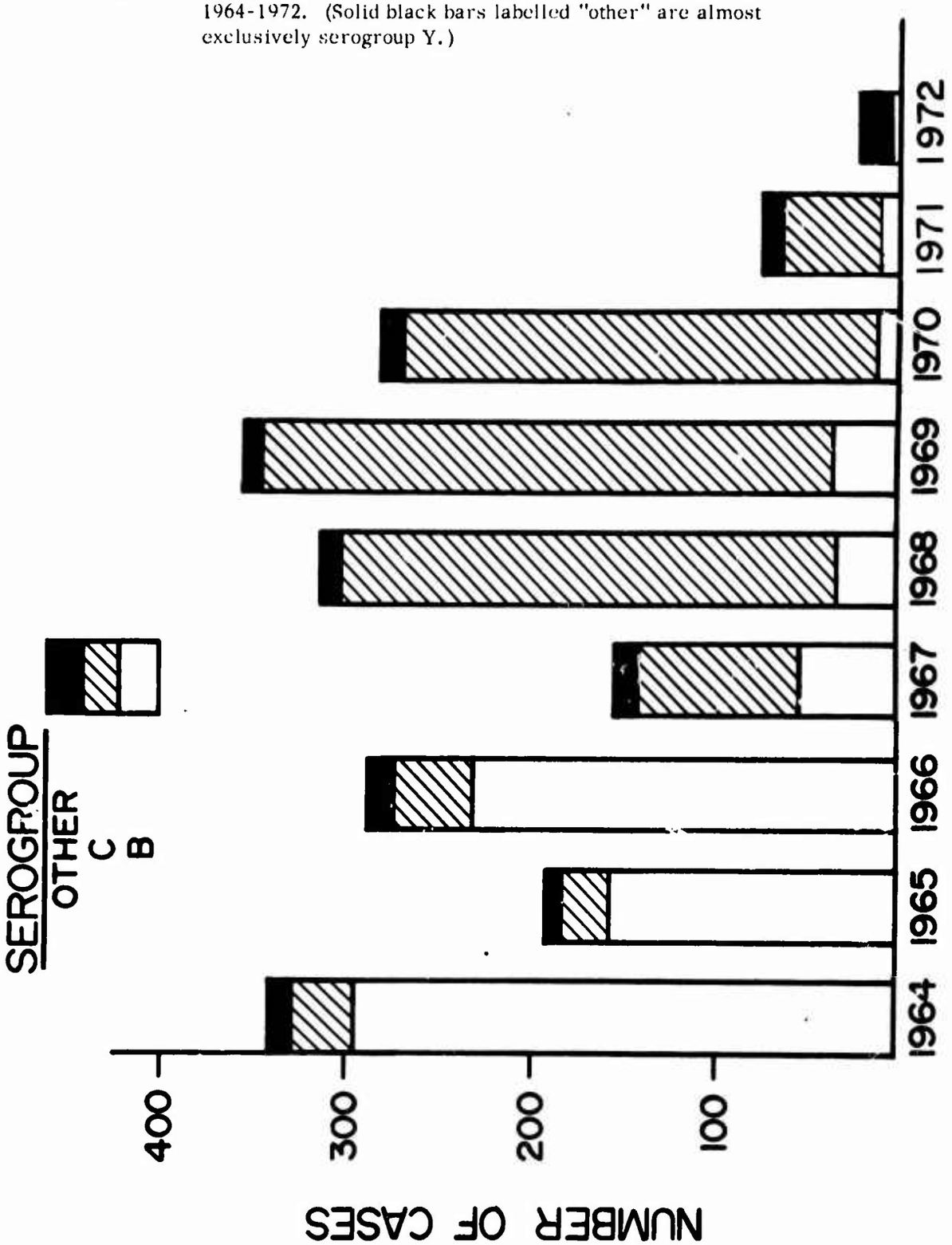
Meningococcal meningitis incidence in U. S. Army active duty personnel has fallen to very low levels beginning in calendar year 1971. This improvement is readily apparent from the data shown in Fig. 1. The number of case strains submitted to WRAIR for confirmation has fluctuated annually with serogroup B strains predominating in 1964-1966, then sharply diminishing and being replaced by a veritable epidemic of serogroup C from 1967-1970. Over 90 percent of the group C strains were resistant to sulfadiazine. Serogroup C strains have not been recognized in active duty meningitis patients all during 1972 and continuing through May 1973. A further breakdown of strains recovered in 1971-1973 is shown in Table 1 from which it is also apparent that serogroup Y is the most frequent disease producer and serogroup B strains have not been a problem in active duty personnel.

Table 1. Meningococcal meningitis: Reported cases and serogroups - 1971-1972-1973.

		Total reported cases	Serogroup of strains tested				
			B	C	Y	Others	Total
Army Active Duty -	1971	123	8	57	11	0	76
	1972	32	1	0	20	0	21
	1973*		1	0	7	Gr 135	9
Army Dependents	1971	**	3	11	1	0	15
	1972	**	6	6	2	0	14
	1973*	**	5	1	0	0	6

* Through 31 May
** No data available

Figure 1: Serogroup distribution of meningococcal case strains, 1964-1972. (Solid black bars labelled "other" are almost exclusively serogroup Y.)



Of interest, strains recovered from dependents (which are not consistently submitted to the reference laboratory) showed a serogroup distribution totally different from the active duty cases; namely, groups B and C were most common.

These changes are attributed to a number of factors: 1) There has been a reduction in the number of training centers as well as a reduction in size of the recruit population. 2) There has been routine use of group C polysaccharide vaccine beginning in October 1971 and continuing through the current period. 3) Group B disease has not emerged in epidemic form (probably not due to protection conferred by the group C vaccine which is highly specific). 4) Group A strains continue to be rare in the continental U. S. 5) The different patterns of serogroup distribution in active duty and dependent cases is evidence that there is little transmission from recruits to the other population.

Meningococcal nasopharyngeal carrier data has been collected at only two recruit training centers in 1972 and 1973. Intermittent surveys at Fort Ord, CA have revealed that carrier rates in 6th or 7th week trainees varied from 35 to 75 percent, group C strains were exceedingly rare, group Y organisms were most common, accounting for from 18 to 61 percent of the isolates and group B strains were 16 to 46 percent of the total recovered. Nevertheless, no group B illnesses were documented at Fort Ord. Fort Leonard Wood, MO surveys showed a different picture - in 1972 total carrier rates ranged from 51 to 71 percent and as high as 80 percent in February 1973. In different companies between 2 and 48 percent of the men carried group B strains, group C carriers varied from 5 to 28 percent of the men tested. Group Y strains colonized from 5 to 33 percent of the men until February and March 1973 when 44 and 47 percent of men tested were Y carriers.

These results suggest that group C vaccine continues to be effective and that neither B nor Y strains have become epidemic in the recruit populations despite continuing high carrier rates.

Acknowledgement: The carrier survey data was kindly provided by the Microbiology Departments of the Fifth and Sixth U. S. Army Laboratories.

2. Antibody response to meningococcal polysaccharide vaccines.

Over the past four years 19 different lots of group C polysaccharide vaccine and five different lots of group A polysaccharide vaccine have been tested for their immunogenicity in man. For each lot tested groups of 20-50 men received 50 micrograms of vaccine subcutaneously. Sera were obtained prior to and two weeks postvaccination. The analytical and serologic methods used in these studies were Sepharose 4B chromatography for the estimation of molecular size, the radioactive antigen binding assay[1] and the indirect hemagglutination (IHA)

test for measuring the antibody response[2].

Results have shown that the radioactive antigen binding assay is preferred to the IHA test as a measure of antibody response. Group C meningococcal vaccines have been highly stable when stored at 4°C in powdered form. All lots of group C vaccine tested to date have been of equal potency; with molecular weight varying from 520,000 to 2.2 million. Group A polysaccharides have been found unstable after two years storage at 4°C. Optimum antibody response to the group A vaccines appears to be directly related to the molecular size of the preparation (Fig. 2).

Acknowledgement: The following non-WRAIR personnel participated in all of the above studies: LTC Creed D. Smith, Chief, Bacteriology Department, Sixth USA Medical Laboratory, Fort Baker, CA; COL H. A. Leighton, Chief, Preventive Medicine Activities, Fort Ord, CA; Members of the Sixth USA Medical Laboratory Respiratory Disease Investigation Team; Mr. Angus Hull, Sixth USA Medical Laboratory, Fort Baker, CA.

3. Relationship between immunoglobulin allotypes and antibody response to meningococcal infection and polysaccharide vaccination.

There are many examples of genetic differences in susceptibility to infection. With the collaboration of Dr. M. S. Schanfield of the Immunoglobulin Typing Laboratory of the University of California, San Francisco, Gm allotypes were determined on sera from patients with meningococcal disease as well as subjects who received group C polysaccharide vaccine. The distribution of Gm phenotypes of the patients with disease was compared with that expected in a normal population. The results of Gm typing of vaccinated subjects were tabulated on the basis of degree of antibody response to the vaccine as measured by radioactive antigen binding assay (RABA)[1].

The distribution of Gm phenotypes of 112 subjects who received group C polysaccharide vaccine is shown in Table 2. There was no striking difference in the magnitude of the antibody response among the various phenotypes (which represent Caucasian phenotypes only; non-Caucasian phenotypes were deleted from the analysis since only a dozen such samples were identified). Thus, very low or very high antibody response was not correlated with any particular Gm phenotype. Although there were differences in the mean RABA levels between phenotypes, there were extremely large variances found and, therefore, it is concluded that the ability to respond to meningococcal polysaccharide vaccine is random.

The distribution of immunoglobulin allotypes among 29 individuals with meningococcal disease is shown in Table 3. Although the number of cases is small there is no evidence for a predisposition of any particular genetic phenotype to acquire systemic illness.

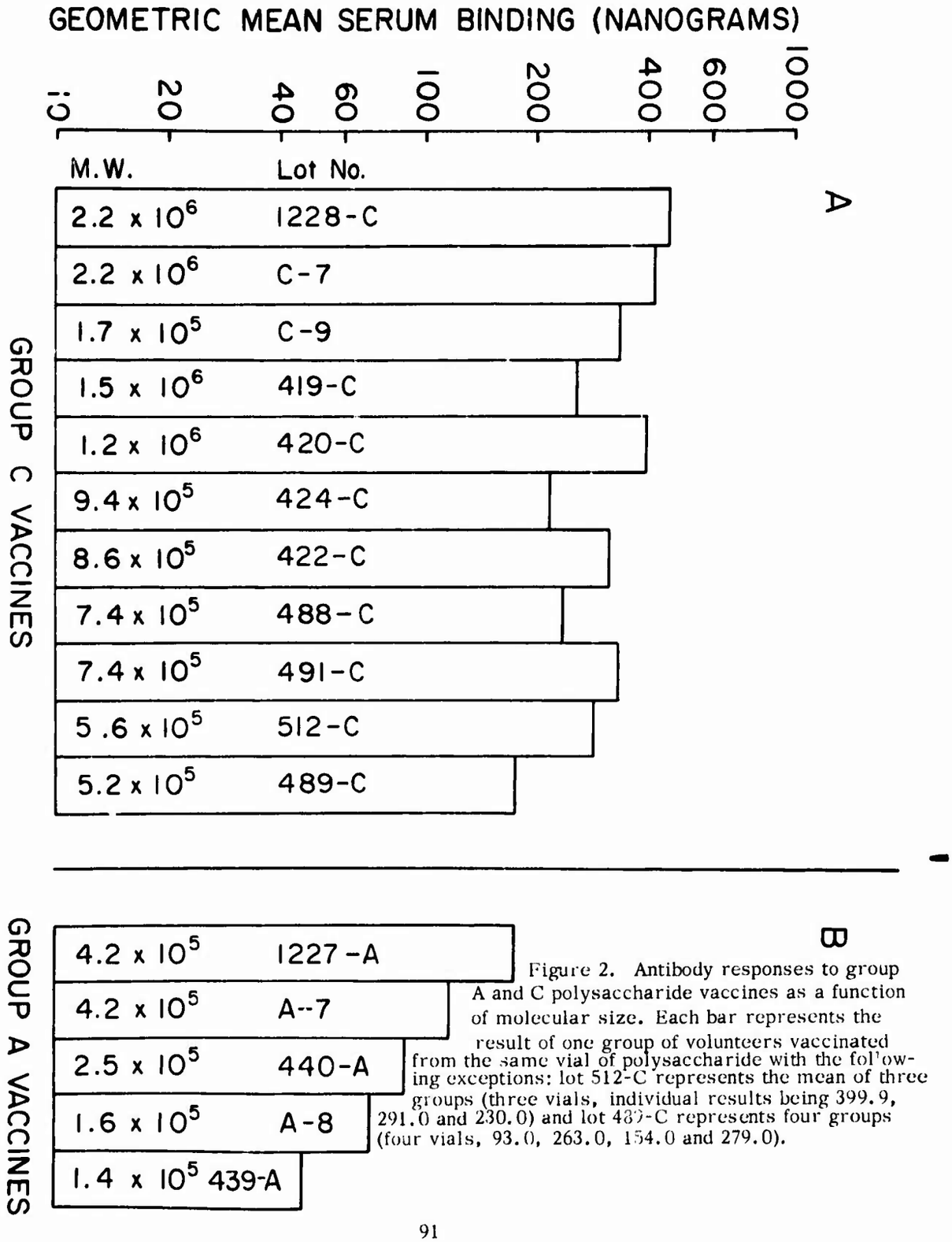


Figure 2. Antibody responses to group A and C polysaccharide vaccines as a function of molecular size. Each bar represents the result of one group of volunteers vaccinated from the same vial of polysaccharide with the following exceptions: lot 512-C represents the mean of three groups (three vials, individual results being 399.9, 291.0 and 230.0) and lot 489-C represents four groups (four vials, 93.0, 263.0, 154.0 and 279.0).

Table 2. Distribution of Gm phenotypes by C-RABA titers.

Phenotype	C-RABA titer range				Total subjects
	0- * 200	201- 400	401- 800	<900	
azgRay	3**	-	3	-	6
axzgRay	1	-	2	-	3
azfgbRayn	3	3	3	4	13
azfgbRay-	4	3	5	4	16
axzfgbRayn	4	2	-	3	9
axzfgbRay-	1	1	-	2	4
fbRayn	18	9	8	15	50
fbRay-	6	2	1	2	11
Total	40	20	22	30	112

* Nanograms of antigen bound

** Number of subjects

Table 3. Immunoglobulin allotypes among patients.

Phenotype	No. of patients with B-Disease	No. of patients with C-Disease
Gm - a,z,g;Ray;Am(1)	0	1
a,x,z;g,Ray;Am(1)	0	0
a,z,f;b,g,Ray;n;Am(1)	2	1
a,z,f;b,g,Ray;-;Am(1)	0	1
a,x,z,f;b,g,Ray;n;Am(1)	0	1
a,x,z,f;b,g,Ray;-;Am(1)	0	1
f;b,Ray;n;Am(1)	5	8
f;b,Ray;-;Am(1)	2	2
Total	9	15
a,z,f;b,s,t,Ray;n;Am(1)*	0	1
a,z;b,Ray;Am(1,2)**	0	1
Not typable	2	1
Total tested	11	18

* Oriental phenotype

** African phenotype

4. Preliminary studies of a meningococcal cell wall protein vaccine.

Although polysaccharides of serogroups A and C have been shown to be immunogenic in man, group B polysaccharides have not been successful[3,4].

Serotype antigens were first described in group C meningococci as a distinct set of antigens different from polysaccharides which could induce bactericidal antibody in sera made from viable organisms[5]. By absorption of the group specific polysaccharide it was possible to identify at least seven antigens which characterized strains of group C organisms. Undoubtedly, further studies will uncover more such antigens. Other studies have shown that strains of serogroups A, B and Y meningococci share these same serotype antigens with group C meningococci[6]. It is felt that these serotype antigens account for the cross reactivity seen when patients who acquire meningococcal infection develop bactericidal antibodies to other serogroups[6].

In group B meningococci it appears that most organisms have multiple antigenic types. Twelve of these had antigen II in combination with other antigens[6]. Of interest, the epidemic of meningitis which had occurred over the past five years has been due to group C strains containing the type II antigen[7].

Earlier studies at WRAIR showed that antigen I and II could be extracted from organisms and that they were protein in nature[8].

Strain B-11 (99M) has been found to contain antigens II, III, IV and IX. Therefore, this strain was used in this initial study of the feasibility of producing a meningococcal protein vaccine.

Three prototype vaccine preparations have been prepared. The extraction procedure used for each is slightly different. By our chemical and serological assays these preparations are quite similar and relatively pure.

Preparation of vaccines:

Protein antigens were all derived from 21 hr cultures of strain B11. Preparations A and K were extracted from the heat killed (60°C x 30 min) sedimented organisms, while preparation Z was derived from the culture medium supernatant.

Preparation A was obtained by extraction with 2.5 percent buffered saline at room temperature. The extract was dialyzed against distilled water, centrifuged to remove insoluble material, and heated in 8 percent trichloroacetic acid (TCA) at 100°C for 15 min to remove nucleic acid. The precipitate was then washed with distilled water, solubilized in dilute NaOH and neutralized.

Preparation K was obtained by an aqueous ether extraction. The aqueous phase was recovered, freed of residual ether and centrifuged to remove particulate matter. The supernatant was then heated at 100°C for 30 min and the precipitate discarded. The cooler supernatant was brought to pH 2.5 and the resulting precipitate collected and resolubilized at pH 10. Nucleic acid was removed by heating in 8 percent TCA as described above for preparation A.

Preparation Z was obtained from a 20-fold concentrate of the culture medium supernatant by precipitation with ethanol to 70 percent v/v. That portion of the precipitate which was soluble in dilute NaOH (pH 11) was treated with 25 percent TCA at 22°C. The precipitate was collected, heated at 100°C in 8 percent TCA for 15 min, and washed with distilled water. After solubilization in dilute NaOH the solution was neutralized and centrifuged to remove any particulate material.

Each preparation was adjusted to a concentration of 1 mg/ml, filter sterilized, dispensed in 2 ml aliquots and lyophilized. For use, vaccines were rehydrated with 20 ml saline. Each vaccine was tested and found sterile and free of animal toxicity and rabbit pyrogenicity.

Results:

Rabbits inoculated seven times over a three week period with a 50 microgram dose intravenously of any of the three vaccines responded with antibodies as shown in Table 4. Bactericidal and antiprotein HA antibodies increased significantly. Another group of rabbits which received lyophilized and then rehydrated material from the same lots responded equally well (the doses in the latter group were only 23 to 34 micrograms). Of interest, three rabbits which were tested for LPS antibodies showed rises to that antigen also.

Another experiment to determine the minimal dose necessary to stimulate antibodies in rabbits is shown in Table 5. A single injection was ineffective in stimulating antibodies but a second injection of both dosages induced high titers of antibodies.

Two volunteers (MSA,DK) were administered 10 micrograms of K protein (0.1 ml) intracutaneously. Local erythema, induration and tenderness began in four hours and reached peak size (erythema) at 48 hrs measuring 6x3 cm diameters in DK and 3.5x4 cm in MSA. No systemic symptoms occurred; reactions faded over the next day or two but slight discoloration was still apparent after seven days. On day two, each volunteer received the same vaccine (40 micrograms subcutaneously) in the deltoid region. At 24 hrs erythema (3.5x3 cm) and slight tenderness were noted in DK; nontender erythema (1.1x1.8 cm) occurred in MSA. Three weeks later booster doses of 50 mcg subcutaneously resulted in local erythema again (3x4 cm in DK; 2x3.5 cm in MSA) which persisted about two days.

Table 4. Bactericidal antibody titers of rabbit sera following immunization with meningococcal protein vaccines.

Vaccine preparation	Rabbit No.	Reciprocal of antibody titer					
		Bactericidal titer*		Protein HA		LPS HA	
		Pre	4 week	Pre	4 wk	Pre	4 wk
A	348	0**	320	0+	640	10	80
A	336A	0	1600	0	2560		
A	336	0	1600	0	1280		
K	298	0	320	0	1280		
K	337	0	1600	0	1280	<10	80
Z	317	0	320	0	2560		
Z	344	0	1600	0	2560		
Z	340	0	1600	0	640	<10	20
Z	342	0	1600	0	1280		

* Test organism 99M (group B meningococcus)

** 0 = <40

\+ 0 = <20

Table 5. Antibody responses in rabbits to Z protein given as two intravenous injections three weeks apart.

Dose group	No. of injections	Serum titers 1 week after injection*	
		Protein HA	Bacteridical (radioactive)
50 mcg	1	1	0
	2	4.25	4.25
250 mcg	1	2	0
	2	6.25	>5.25

* Geometric mean tube rise (4 rabbits each group), dilutions began at 1:10.

Antibody response (RBT, Table 6) showed high unchanging titers in both volunteers after initial and booster vaccinations. The other antibody tests were performed at a later date and showed a suggestive increase in titer by HA test in subject DK.

One month later two further volunteers (BLB and JW), whose RBT titers were much lower, were inoculated with K vaccine (50 mcg, subcutaneously). BLB developed local induration, slight tenderness and erythema (3x4 cm, peak size at 32 hrs) which disappeared by 48 hrs. JW had no reaction at the injection site. Booster inoculation three weeks later induced a similar local reaction in BLB (3x2 cm at 24 hrs) which disappeared a day later. Again, JW did not react. Both subjects failed to show RBT titer increase but both showed suggestive rises by HA test. (In these two subjects, the initial three serum specimens showed 3-4+ agglutination in the first two dilutions and then 2+ reactions through tube 8 whereas the later sera gave 3-4+ agglutination throughout eight tubes. Therefore, it is uncertain that the change is significant.)

Four more subjects received either A or Z vaccine preparations; initial injection consisting of 10 mcg intracutaneously and 40 mcg subcutaneously one day later. Dermal reactions to 10 mcg were similar to those of subjects DK and MSA above, largest diameter of erythema varying from 2.5 to 6 cm at 24 hrs with marked diminution in 48 hrs. WB had a moderate local and systemic reaction to the 40 mcg SC dose with aching of the arm, local tenderness and swelling, a pea sized axillary node noted at six hrs but no fever. Oral analgesic was taken and symptoms had abated within 22 hrs. For the booster dose only 10 mcg SC was administered to this subject and only transient local tenderness and erythema developed. Antibody studies (Table 6) revealed only suggestive HA rises in WB and HN.

Discussion:

The lack of consistent and significant increases in antibodies in these eight volunteers was disappointing. Volunteers had been selected to include a range of prevaccination antibody levels. Further serum testing will be undertaken if and when a radioactive protein precipitation assay is developed. One technical difficulty was noted in that thawing of the frozen vaccines induced a turbidity in what had been a completely clear solution prior to freezing. That this alone did not reflect a major change in potency can be inferred from the fact that rabbits receiving the same material responded dramatically. Of course, on a weight basis, the rabbits received 10-20 fold more antigen and had been injected intravenously. Although rabbit pyrogenicity tests showed the vaccines to contain less than 0.4 percent endotoxin, the fact that each rabbit tested developed an increase in LPS antibodies suggests that trace amounts of LPS were present and may have been responsible for the systemic reaction in subject WB.

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Table 6. Antibody response of 8 volunteers to protein vaccines.

Subject		Serum date (weeks) 0	1	2	3	4	5	6	7	8	9
DK	Vaccine/dose*	K/50			K/50						
	Protein-HA**	9	9	≥11	10		≥11	≥11			
	Cidal †	10,000		10,000			4,000				
	IFA ††	8	8	8	8		16	4			
MSA	Vaccine/dose	K/50			K/50						
	Protein-HA	9	9	9	9		9	9			
	Cidal	10,000		10,000			4,000				
	IFA	8	16	8	8		16	16			
BLB	Vaccine/dose	K/50			K/50						
	Protein-HA	2	2		2	8	8		8		
	Cidal	32	16	32	32	32	32				
	IFA	<2	<2	<2	<2		<2		<2		
JW	Vaccine/dose	K/50			K/50						
	Protein-HA	2	2		2	8	8				
	Cidal	8	4		8	8	8				
WB	Vaccine/dose	A/50			A/10						
	Protein-HA	0	1	3	2	2					
	Cidal	16			16	16	16				8
K	Vaccine/dose	A/50			A/50						
	Protein-HA	1	1	2	1	2					
	Cidal	<1			<1	<1	<1				
HN	Vaccine/dose	Z/50			Z/50						
	Protein	1		4	3	1					
	Cidal	16			16	16	16			16	

Table 6. (Continued)

Subject		Serum date (weeks)									
		0	1	2	3	4	5	6	7	8	9
ECT	Vaccine/dose	Z/50			Z/50						
	Protein-HA	3	2	3	2	2					
	Cidal	16			16	16	16			16	

* Three lots of vaccine used (K, A, Z), 50 microgram dose, SC using plastic syringes.

** Number of serial 2-fold dilutions.

† Radioactive bactericidal test, reciprocal of serum dilution, test organisms 99M.

†† Reciprocal of indirect fluorescent antibody titer, uses fluorescein tagged anti-human IgG versus 99M organism as antigen.

It is hoped to resolve these problems by extracting the protein antigen in a gentler fashion and by improving the purification. Subsequent preparations will have to be tested in humans in smaller and larger quantities to resolve the question of optimum dosage.

Acknowledgement: These studies were initiated and carried out by Dr. Dennis L. Kasper during his duty assignment in this laboratory. The vaccines were prepared in the Department of Biologics Research with the assistance of P. Altieri, S. Berman and J. Lowenthal.

Recommendations:

The routine use of group C meningococcal polysaccharide vaccine in Army recruits has continued to be an effective preventative against group C disease. Group Y disease has been occurring with increasing incidence but remains a rather minor problem in that only 20 such cases were proven in calendar year 1972. Nevertheless, this situation bears watching. Physico-chemical specifications for potent vaccines are not yet available but molecular sizing by Sepharose 4B chromatography appears promising. Group C vaccines have been very stable when stored as powder, whereas group A polysaccharides appear to deteriorate within two years under identical conditions of storage. Until this problem is solved stockpiling group A vaccines for use in the future would be a waste of resources. Attempts to determine a genetic basis for the variability of immunologic response to vaccination have proven unsuccessful in terms of immunoglobulin Gm typing.

Preliminary studies of meningococcal cell wall protein vaccines have shown these antigens to induce antibodies in rabbits quite readily. However, human immunologic responses were minimal. Further studies are indicated to improve the extraction and purification methods and to determine the optimum dose for man.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 181 Development of a meningococcal immunizing agent

Literature Cited.

References:

1. Brandt, B. L., Wyle, F. A., and Artenstein, M. S. A radioactive antigen binding assay for Neisseria meningitidis polysaccharide antibody. J. Immunol. 108:913-920, 1972.

2. Artenstein, M. S., Brandt, B. L., Tramont, E. C., Branche, W. C., Jr., Fleet, H. D., and Cohen, R. L. Serologic studies of meningococcal infection and polysaccharide vaccination. J. Infect. Dis. 124:277-288, 1971.

3. Gotschlich, E. C , Goldschneider, I., and Artenstein, M. S. Human immunity to the meningococcus. IV. Immunogenicity of group A and group C meningococcal polysaccharides in human volunteers. J. Exp. Med. 129:1367-1384, 1969.

4. Wyle, F. A., Artenstein, M. S., Brandt, B. L., Tramont, E. C., Kasper, D. L., Altieri, P. L., Berman, S. L., and Lowenthal, J. P. Immunologic response of man to group B meningococcal polysaccharide vaccines. J. Infect. Dis. 126:514-522, 1972.

5. Gold, R., and Wyle, F. A. New classification of Neisseria meningitidis by means of bactericidal reactions. Infec. & Immun. 1:479-484, 1970.

6. Kasper, D. L., Winkelhake, J. L., Brandt, B. L., and Artenstein, M. S. Antigenic specificity of bactericidal antibodies in antisera to Neisseria meningitidis. J. Infect. Dis. 127:378-387, 1973.

7. Gold, R., Winkelhake, J. L., Mars, R. S., and Artenstein, M. S. Identification of an epidemic strain of group C Neisseria meningitidis by bactericidal serotyping. J. Infect. Dis. 124:593-597, 1971.

8. Wyle, F. A., and Kasper, D. L. Immunochemical studies on serotype antigens of Neisseria meningitidis. (Abstract) Bact. Proc. p. 99, 1971.

Publications:

1. Wyle, F. A., Artenstein, M. S., Brandt, B. L., Tramont, E. C., Kasper, D. L., Altieri, P. L., Berman, S. L., and Lowenthal, J. P. Immunologic response of man to group B meningococcal polysaccharide vaccines. J. Infect. Dis. 126:514-522, 1972.

2. Winkelhake, J. L., and Kasper, D. L. Affinity chromatography of anti-meningococcal antiserum. J. Immunol. 109:824-833, 1972.

3. Kasper, D. L., Winkelhake, J. L., Zollinger, W. D., Brandt, B. L., and Artenstein, M. S. Immunochemical similarity between polysaccharide antigens of E. coli and group B Neisseria meningitidis. J. Immunol. 110:262-268, 1973.

4. Kasper, D. L., Winkelhake, J. L., Brandt, B. L., and Artenstein, M. S. Antigenic specificity of bactericidal antibodies in antisera to Neisseria meningitidis. J. Infect. Dis. 127:378-387, 1973.

5. Monto, A. S., Brandt, B. L., and Artenstein, M. S. Response of children to meningococcal polysaccharide vaccines. J. Infect. Dis. 127:394-400, 1973.

6. Maloney, P. C., Schneider, H., and Brandt, B. L. Production and degradation of serogroup B Neisseria meningitidis polysaccharide. Infect. & Imm. 6:657-661, 1972.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B6444	92 08 07	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
72-07-01	H. Termination	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	183
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Diseases of Military Animals in Southeast Asia (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology 005900 Environmental Bio

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
68 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 72	6	120
	CURRENT 73	0.5	12

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

21. GENERAL USE

Foreign intelligence not considered.

ASSOCIATE INVESTIGATORS

NAME: Hildebrandt, LTC, P. K.

NAME: Binn, Ph.D., L. N. DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Dogs; (U) Tropical Canine Diseases; (U) Tropical Canine Pancytopenia; (U) Ehrlichia canis; (U) Babesia Trypanosoma

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To define, study, diagnose and control known and potential infectious diseases of military dogs in Southeast Asia and other areas of potential military significance. The major effort is directed toward the cause, pathogenesis, treatment and control of tropical canine pancytopenia (TCP) which has jeopardized the operational efficacy of military dogs in SE Asia. Studies are also being conducted on the epidemiology, treatment and control of Babesia and other parasitic infections which are medical problems in military dogs.

24. (U) Conventional methods are employed for epidemiological, pathological and microbiological examinations, and new procedures are developed as needed.

25. This work unit has been consolidated with work unit 056, Diseases of Military Animals, Project 3A062110A830, Biosensor Systems. Progress is reported therein.

*Available to contractors upon originator's approval.

DD, FORM 1498, MAR 88 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 65 (FOR ARMY USE) ARE OBSOLETE.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B6445	72 08 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
72 07 01	H.Termination	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	184
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede with Security Classification Code)

(U) Diseases of Recruit Military Animals (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
68 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 72	1.2	21
FISCAL YEAR CURRENT 73	0.2	3

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D.C. 20012

RESPONSIBLE INDIVIDUAL

NAME: Buescher, COL, E. L.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of Veterinary Medicine

ADDRESS: Washington, D.C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Binn, Ph.D., L. N.

TELEPHONE: 202-576-5381

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Huxsoll, LTC, D. L.

NAME: DA

PII Redacted

21. GENERAL USE

Foreign intelligence not considered.

22. KEYWORDS (Procede EACH with Security Classification Code) (U) Military Dogs; (U) Recruit Dog Diseases; (U) Canine Parainfluenze; (U) Canine Rhinovirus; (U) Canine Coronavirus; (U) Canine Cell Culture

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Procede text of each with Security Classification Code.)

23. (U) To determine and characterize causative agents of infectious diseases occurring in military dogs during their induction and training; to determine how the infections are spread; and to develop effective control measures. Studies on agents associated with acute respiratory disease and diarrheal diseases are specifically emphasized. The potential importance of infectious agents newly found in dogs are evaluated.

24. (U) Conventional epidemiologic and microbiologic technics are employed and new procedures will be developed as needed.

25. This work unit has been consolidated with work unit 056, Diseases of Military Animals, Project 3A062110A830, Biosensor Systems. Progress is reported therein.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6517	73 0/ 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
	A. New	U	U	NA	NA	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	185
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Speciation of biomolecules by mass spectrometry

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002300 Biochemistry 008300 Inorganic Chemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	Cont	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 73	4	100
	CURRENT 74	4	100

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL
NAME: Buescher, COL E. L.
TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of Biochemistry
ADDRESS: Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Beach, LTC, D. J.
TELEPHONE: 202-576-2211
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

ASSOCIATE INVESTIGATORS
NAME: Demaree, LTC G.E.; Kelley, CPT J.E.
NAME: Bass, B.G.,MS; Kazyak, L., BS DA

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Mass Spectrometry; (U) Drug Metabolism; (U) Gas Chromatography; (U) Analytical Chem

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) The technical objective of this work unit is to develop and establish methodology and analytical techniques for the detection, identification and characterization of important biochemical compounds and their principal metabolites using coupled gas chromatography-mass spectrometry (GC-MS).

24. (U) Computer controlled GC-MS systems will be used to definitively characterize and quantitate compounds of biochemical importance for both developmental and research uses. Efforts will be focused on rapid, accurate identification of a variety of compounds and on feasibility of the implementation of these systems for research and practical general laboratory use. Efforts will be concentrated on metabolites of compounds of interest in drug abuse programs and in the study of metabolism of biomolecules both natural and foreign to mammalian biosystems. A library of mass spectra of drugs of abuse of military significance will be developed.

25. (U) 72 07 - 73 06 A computerized GC-MS system has been installed. Personnel training and checkout of the operating system are in progress. An organic synthetic laboratory for synthesis of standards was established. Synthesis of standards for cocaine metabolites was completed. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72 - 30 Jun 73.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 185 Speciation of biomolecules by mass spectrometry

Investigators.
Principal: COL Charles R. Angel, MSC
Associate: LTC Douglas J. Beach, MSC; LTC Gale E. Demaree, MSC; Clarence E. Emery, B.S.; Laurence R. Hilpert, B.S.; Leo Kazyak, B.S.; CPT James A. Kelley, MSC; SP4 George A. Locko, B.S.; Robert C. Permisohn, M.S.; SP4 Steven J. Weise, B.S.

The objective of this work unit is to develop and establish methods and analytical techniques for the detection, identification, characterization and quantitation of important biochemical compounds and their principal metabolites using coupled gas chromatography-mass spectrometry systems.

During this report period, work progressed in these areas:

1. Morphine derivatives.

2. Methaqualone metabolites.

3. Cocaine metabolites.

4. Heroin metabolites.

1. Morphine derivatives.

Trimethylsilyl ether (TMS) derivatives of morphine to increase detection sensitivity by GLC were investigated. Although the TMS derivatives increased the sensitivity of the procedure for morphine, the extracts of some hydrolyzed urine specimens contained unknown materials whose TMS derivatives had retention times near that of the morphine derivative. This causes serious interference with morphine detection.

2. Methaqualone metabolites.

A study was initiated to develop a method for detection of methaqualone in urines of users. Urine from volunteers who had ingested a single 250 mg tablet was collected for 48 hours and analyzed by ultraviolet spectrophotometry (UV), GLC, and MS. There are no spectral distinctions by UV among the drugs and its metabolites. These can be distinguished by GC-MS. The maximum amount of unchanged methaqualone

appeared in the urine at 2 to 4 hours after ingestion. The metabolites identified in urine were essentially the same as those reported by R. Bonnichsen and co-workers. The metabolites as reported by Bonnichsen were also isolated in our laboratory.

One other metabolite, a methoxy derivative was reported by Bonnichsen. Our mass spectral data suggest the possibility of the formation of this derivative, but until additional studies are complete, this metabolite must be considered as tentative. Preliminary data indicate the presence of a dihydroxy methaqualone, which has not been reported previously. The positions of the hydroxyl groups are not certain but attachment to the o-tolyl ring is likely.

Efforts to isolate large amounts of all methaqualone metabolites are in progress for infrared and nuclear magnetic resonance spectral analyses.

Separation of the metabolites using capillary columns for GLC is being investigated in the hope that with greater column efficiency and resolution metabolites can be resolved.

3. Cocaine metabolites.

Cocaine and its metabolites, ecgonine and benzoylecgonine were analyzed by GC-MS in urine, blood and spinal fluid of monkeys receiving cocaine.

Ecgonine and benzoylecgonine were synthesized for standards by the hydrolysis of cocaine under appropriate conditions. N-butylboronate esters and BSA esters of these compounds were synthesized for GC-MS analysis.

Serial urine specimens were obtained for each of two monkeys after I.V. injection of 1 mg/kg of cocaine. These urine samples contained ecgonine but not cocaine or benzoylecgonine. Ecgonine was detected up to 61 hours post-injection. Quantitation of the ecgonine present in each sample is proceeding. Blood and cerebral spinal fluid samples will also be analyzed.

4. Heroin metabolites.

Collaborative studies with the Division of Neuropsychiatry are being conducted to develop an analytical system employing radioimmunoassay combined with chromatographic separation techniques and mass spectrometry coupled to GLC for identification and quantitation of drugs of abuse and their metabolites in biological fluids and tissues from experimental animals. Antibodies to morphine are being evaluated. The instrumentation for GC-MS has been installed and is undergoing technical evaluation.

Literature Cited.

References:

1. Bonnichsen, R., Claes-Goran, F., Carmen, N., and Ryhage, R.: Identification of methaqualone metabolites from urine extract by gas chromatography-mass spectrometry. Clin. Chim. Acta 40: 309-318, 1972.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION* DA OB 6519	2. DATE OF SUMMARY* 73 07 01		REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SUM'RY	4. KIND OF SUMMARY A. New	5. SUMMARY SCTY* U	6. WORK SECURITY* U	7. REGRADING* NA	8a. DISP'N INSTR'N NL	8b. SPECIFIC DATA-CONTRACTOR ACCESS* ☒ YES ☐ NO	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	190
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede with Security Classification Code)*

(U) Effect of Anti-malarial Drugs on Dental Plaque

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:*

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 73	0.5	20
	CURRENT 74	0.5	20

19. RESPONSIBLE DOD ORGANIZATION

NAME:* Walter Reed Army Institute of Research

ADDRESS:* Washington, DC 20012

RESPONSIBLE INDIVIDUAL

NAME: Buescher, COL E. L.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME:* Walter Reed Army Institute of Research
Division of Medicinal Chemistry

ADDRESS:* Washington, DC 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME:* Kinnamon, K. E., LTC

TELEPHONE: 202-576-2292

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

ASSOCIATE INVESTIGATORS

NAME: Atherton, B. T., 1LT

NAME: Wolf, C. R., 2LT DA

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Procede EACH with Security Classification Code)

(U) Test System; (U) In vitro; (U) In vivo; (U) Dental Plaque; (U) Periodontal Disease

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Procede text of each with Security Classification Code.)

23. (U) To design and develop in vitro and in vivo test systems for the evaluation of chemical compounds as prophylactic and therapeutic agents against diseases of major military significance. Infections such as malaria, which render troops ineffective for extended periods, receive principal emphasis.

24. (U) Appropriate in vitro and animal pilot models are designed to meet a specific need for testing chemical compounds. These models are expanded into operating test systems in-house and utilized to provide guidance to contractors when testing is to be done under contract. When necessary, modifications to existing test systems are designed and developed.

25. (U) 72 07 - 73 06 Bacterial deposits on the crowns and roots of teeth have been implicated in periodontal disease for many years. The plaque-forming characteristics of certain bacteria are used to determine if drugs are effective in prevention and/or elimination of the disease. Several compounds having known antimalarial activity have shown antibacterial activity in a preliminary in vitro assay. These same compounds were found not to be effective in preventing the formation of plaque in hamsters when the drugs were introduced daily into the oral cavity in an aqueous suspension. Some of the compounds have been incorporated into the hamster diets. None so far tried, have prevented plaque formation. Other compounds showing in vitro antibacterial activity are being evaluated. These investigations have been previously reported under Project 3A663713D829, Task 00, Work Unit 122, and have been done with the technical advise of Dr. Paul H. Keyes, National Institute of Dental Research, NIH. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72-30 Jun 73.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 190 Effect of antimalarial drugs on dental plaque

Investigators.

Principal: LTC Kenneth E. Kinnamon, VC

Associate: 2LT Charles R. Wolf, MSC; 1LT James H. Smith, MSC

Bacterial deposits on the crowns and roots of teeth have been implicated in periodontal disease for many years. It was noted that in servicemen returning from areas where antimalarial drugs were routinely taken the incidence of dental plaque was lower. It was of interest to determine if certain selected compounds with known antimalarial properties would influence the bacteria responsible for plaque formation. Initially an in vitro system employing nutrient agar was used. Many of the antimalarial compounds employed showed antibacterial activity. Next drugs showing in vitro activity were dissolved or suspended in a suitable vehicle and delivered into the oral cavity of hamsters once daily by pipette at a dose of 25 mg/day. Using this method no effect on the development of periodontal disease was observed. Present studies involve the incorporation of the antimalarial compounds into the hamster diet. Chloroquine (WR 1544) and primaquine (WR 2975) have been shown to be inactive at a dose level of:

1. Chloroquine 100 mg/kg of diet ad libitum
2. Chloroquine 500 mg/kg of diet ad libitum
3. Chloroquine 1000 mg/kg of diet ad libitum
4. Primaquine 15 mg/kg of diet ad libitum
5. Primaquine 75 mg/kg of diet ad libitum
6. Primaquine 150 mg/kg of diet ad libitum
7. Chloroquine (100 mg/kg) + Primaquine 15 mg/kg of diet ad libitum
8. Chloroquine (500 mg/kg) + Primaquine 75 mg/kg of diet ad libitum
9. Chloroquine (1000 mg/kg/ + Primaquine 150 mg/kg of diet ad libitum

The data are being evaluated to determine if it is advisable to continue these studies.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6520	73 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
	A. New	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	63713A	3A061101A91C	00	191
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Immunosuppressive Activity of Medicinal Compounds

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	CONT	DA	In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 73	2	50
	CURRENT 74	2	60

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D.C. 20012

RESPONSIBLE INDIVIDUAL

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20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of Medicinal Chemistry

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PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Kinnamon, K. E., LTC

TELEPHONE: 202/576-2292

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

ASSOCIATE INVESTIGATORS

NAME: Atherton, B. T., 1LT

NAME: Wolf, C. R., 2LT DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Immunosuppression; (U) CFU's; (U) Spleen Colony Assay; (U) Simonsen Assay; (U) Bone Marrow; (U) Allogeneic

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To conduct testing to evaluate the immunosuppressive activity of various medicinal compounds.

24. (U) Certain chemical agents which are being tested primarily for certain properties, e.g., radiation protective properties, may also have immunosuppressive properties. These immunosuppressive properties in some cases may serve to guide the development of potential drugs.

25. (U) 72 07 - 73 06 To assess the immunosuppressive activity of various medicinal compounds, two biological systems will be employed. These are the spleen colony assay using allogeneic bone marrow and the Simonsen infant mouse assay using spleen cells. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72 - 30 Jun 73.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 191 Immunosuppressive Activity of Medicinal Compounds

Investigators:

Principal: LTC Kenneth E. Kinnamon, VC

Associate: 2LT Charles R. Wolf, MSC

The roles of humoral and cellular mechanisms in diseases and sickness is well recognized. These roles can be significantly altered by certain chemotherapeutic agents. For example certain agents with radiation protective properties have been found to have immunosuppressive properties. Thus, these compounds administered prior to radiation exposure may not only function in a radioprotective fashion but may also serve as an immunosuppressant and, therefore, enhance the compatibility of foreign bone marrow that may be administered. Too, the roles of immune mechanisms to malaria have not been elucidated. Although serum mediated passive immunity has been well established and cellular factors involved in malaria infection have also been extensively studied the effects that chemotherapeutic agents have on these immune mechanisms has not been well defined. For selected compounds the immunosuppressive activity of these will be determined by the spleen colony assay described by Till and McCulloch (1) and the infant mouse assay of Simonsen (2).

Literature Cited.

References:

1. Till, J. E. and McCulloch, E. A.: A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiation Rsch. 14: 213-222, 1961.

2. Simonsen, M. and Jensen, E.: The graft versus host assay in transplantation chimaeras. In: A Symposium on Biological Problems of Grafting, edited by F. Albert and P. B. Medawar. Oxford: Blackwell, 1957, p. 214-238.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6521	73 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
	A. New	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	192
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede with Security Classification Code)

(U) Pharmacological effects of WR 2823 and other candidate anti-hypotensive drugs (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012600 Pharmacology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	CONT	DA	In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER: NA

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 73	1	30
FISCAL YEAR CURRENT 74	1.5	45

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL

NAME: BUESCHER, COL, E.L.

TELEPHONE: 202/576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Division of Medicinal Chemistry
Washington, DC 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

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TELEPHONE: 202/576-3387

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: EINHEBER, Dr., A.

NAME: MINER, LTC, L.C. DA

PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Procede EACH with Security Classification Code)

(U) Pharmacology; (U) Drugs; (U) Medicinals; (U) Hypotension; (U) Shock

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Procede text of each with Security Classification Code.)

23. (U) Research is directed toward investigating the pharmacology of promising medicinal agents, drug interactions, developing and refining animal models for the study of hemorrhagic, septicemic, and traumatic injury-induced hypotension as well as the exploitation of Army procured chemicals in the treatment and prevention of this syndrome. Studies are directed toward determining the mechanisms of action of therapeutic agents as well as the nature and type of chemicals which would be useful in this therapy. The goal of this research is to develop a highly effective, non-toxic drug which would be useful in the treatment or prevention of trauma associated with battlefield injury.

24. (U) Drugs are tested in animal models for effectiveness in preventing or treating experimental hypotension resulting from hemorrhage, endotoxin, traumatic injury, and anaphylactic stress.

25. (U) 72 07 - 73 06 Phase I human tolerance study completed for WR 2823. Pharmacodynamic studies with WR 2823 and WR 149,024 were continued and extended to include the effect on renal function of normotensive, anesthetized dogs. The life-saving effects in treating shock may be due in part to their effects on renal function. WR 149,024 was effective in reducing the mortality of E. coli endotoxin shock in dogs. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 72 - 30 June 73. Studies were formerly reported under work unit number 036, project 3A061102B71P.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.

527-825 O - 74 - 9

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 192 Pharmacological effects of WR 2823 and other candidate anti-hypotensive drugs

Investigators.
Principal: Melvin H. Heiffer, Ph.D.
Associate: LTC L. Miner, Dr. A. Einheber, CPT H. Ellis, III, Dr. R. Rozman, A. Berman

1. Description.

The major efforts of this program are directed to investigating the pharmacology of promising medicinal agents which antagonize hypotension due to such etiological conditions as hemorrhage and endotoxin poisoning as well as other causes. These include elucidation of: drug interactions with autonomic and autocoid receptors; the nature of these receptors; the metabolism of these drugs; and the pharmacology of their metabolites.

Appropriate pharmacological, physiological and biochemical studies are conducted in vitro and in vivo. These studies encompass the acute responses to these medicinal chemicals and their interactions with standard pharmacological and physiological agents. A feature of the capabilities of this department resides in the vast inventory of serially related and diverse chemicals which can be used in detailed pharmacological and screening studies on the nature of drug interactions with biological systems.

2. Human studies.

Phase I studies have been completed with WR 2823. The reports from these studies are currently being evaluated.

With receipt of formulation details, the IND for WR 149,024 is at the stage of requesting permission to begin human tolerance trials.

3. WR 2823 antagonism of norepinephrine-induced contractions in the isolated rabbit aorta.

a. Background:

In the present synthetic route of S-2-[5-(aminopentyl)amino] ethyl phosphorothioic acid (WR 2823), it is impossible to avoid the formation of small amounts of its symmetrical disulfide, 1,18-diamino-6,13-diaza-9,10-dithiaoctadecane (WR 149,024). The amount of WR 149,024 in the four lots of WR 2823 varied from 0.5 to 13%.

Since there is evidence that WR 2823 is biotransformed to WR 149,024 and since the symmetrical disulfide is approximately four times as potent an alpha adrenergic blocking agent as WR 2823, we sought to determine whether WR 2823 per se was an alpha adrenergic blocking agent.

b. Methods:

To test this hypothesis, isolated rabbit thoracic aorta strips were suspended in a muscle bath and experimental procedures were carried out according to the methods described by Demarco et al. (1971) and Furchgott (1960) with the following modifications: arteries were allowed to relax for 3 hours before constructing cumulative concentration-response curves; norepinephrine was added to obtain concentrations up to 3×10^{-7} Molar during the control period; and a Lineweaver-Burk plot was drawn to estimate the maximum response.

Norepinephrine, up to 10^{-5} Molar, was added to the bath in the presence of the antagonist. All doses of norepinephrine were allowed to remain in contact with the arteries for 7 minutes before adding the next concentration, except for 10^{-5} M which was kept in contact for 10 minutes. Chromatographic separations were made at the beginning and end of the experiment to preclude the possibility that WR 2823 was converted to WR 149,024 in the water bath.

c. Analysis:

The following parameters among each group were compared:

i. $\text{Log}_{10} \frac{A_b}{A_o}$, where A_b is the concentration of norepinephrine required to give 50% of the maximum response in the presence of the antagonist and A_o is the concentration of norepinephrine required to given 50% of the maximum response in the absence of the antagonist.

ii. The variances between and within the groups.

iii. The means of the groups, with significance set at the 99% level.

d. Design:

Five groups were used:

Group I - Saline vehicle was added.

Group II - The antagonist was WR 2823B, which contained 13% of WR 149,024.

Group III - The antagonist was WR 2823AB, which contained 0.5% of WR 149,024.

Group IV - The antagonist used was WR 149,024 in a concentration equal to that found in Group II (13% = 5.12×10^{-5} Molar).

Group V - The antagonist used was WR 149,024 in a concentration equal to that found in Group III (0.5% = 1.96×10^{-6} Molar).

e. Results:

The following data were obtained:

Group		Mean $\text{Log}_{10} \frac{A_b}{A_o}$	S.E.
I	saline	-0.037	± 0.0222
II	WR 2823B (= 13%)	0.478	± 0.0730
III	WR 2823AB (= 0.5%)	0.355	± 0.0574
IV	WR 149,024 (= 13%)	0.404	± 0.0870
V	WR 149,024 (= 0.5%)	0.177	± 0.0550

The above data were analyzed for significance according to Tukey's omega procedure (Steel and Torrie, 1960). WR 2823B (Group II), which contained 13% of WR 149,024, was no different from the group treated with that amount of WR 149,024 only. WR 2823B was no different from WR 2823AB, which contained only 0.5% disulfide. However, WR 2823AB antagonized the norepinephrine-induced contractions of the aorta to a greater degree ($P < .01$) than group V, which was treated with 1.96×10^{-6} Molar of WR 149,024 (equivalent to the amount of symmetrical disulfide found in WR 2823AB) only.

Thin layer chromatography revealed that WR 2823AB was not converted to the symmetrical disulfide in the bath during the experiment, confirming the finding of Herrington et al. (1967) who used a different phosphorothioate.

The data are consistent with the hypothesis that WR 2823 per se is an alpha adrenergic receptor blocking agent.

4. Further investigations on the pharmacology of WR 2823 in vivo in dogs.

The purpose of this study was to determine whether WR 2823 per se was an alpha adrenergic blocking agent in vivo or whether its effect on the receptors was due to the unavoidable trace amounts (0.5%) of its symmetrical disulfide, WR 149,024.

a. Approach:

Dogs were anesthetized with 30 mg/kg of sodium pentobarbital and prepared for intravenous infusions, as well as measurement of arterial blood pressure, aortic blood flow, hind limb blood flow, EKG and respiratory exchange. Dose response curves to epinephrine, norepinephrine and angiotensin were constructed. The dogs were divided into two groups. One group received 50 mg/kg of WR 2823AB i.v., which contained 0.5% (equal to 0.25 mg/kg) of its symmetrical disulfide, WR 149,024. The other group was given i.v. doses of WR 149,024 ranging from 0.25 mg/kg (equivalent to the amount of WR 149,024 contained in WR 2823AB) to 3.75 mg/kg. One hour after the antagonist was administered, dose response curves for the agonists were constructed again.

b. Results:

WR 2823AB was found to attenuate the pressor response to norepinephrine and to convert the pressor effects of epinephrine to depressor responses without inhibiting epinephrine-induced tachycardia or diminishing epinephrine-induced increases in aortic blood flow. On the other hand, WR 149,024, in the doses used, neither attenuated the pressor responses to norepinephrine nor caused epinephrine reversal. Therefore, WR 149,024, in quantities up to 15 times the amount contained in WR 2823AB, was not responsible for the alpha adrenergic blocking action of WR 2823AB in vivo.

5. Dissociation of vascular and gut responses to WR 2823 and WR 149,024.

This study was designed to determine whether gut smooth muscle reacts to the alpha adrenergic blocking action of these two drugs in the same manner as does vascular smooth muscle.

a. Methods:

Dogs were anesthetized with sodium pentobarbital and prepared for i.v. injections and arterial blood pressure measurements. A balloon-tipped catheter was inserted into the duodenum and pressure changes recorded. Isolated guinea pig ileum strips were prepared according to standard methods.

b. Results:

WR 2823 and WR 149,024 in i.v. doses which attenuated the vasopressor effects of i.v. epinephrine and phenylephrine did not

affect the inhibition of gut motility produced by either of these catecholamines. In these dogs, phentolamine, 1 mg/kg, was capable of completely blocking the effects of phenylephrine on the gut and on blood pressure. In addition, graded i.v. doses of phentolamine, phenoxybenzamine and chlorpromazine showed a dose-related ability to produce alpha adrenergic blockade simultaneously in both the gut and the vasculature.

In the isolated guinea pig ileum, neither WR 2823 nor WR 149,024 prevented the inhibition of spontaneous muscle activity caused by epinephrine and phenylephrine.

Thus, WR 2823 and WR 149,024 produced alpha adrenergic antagonism in the vasculature but not in the gut, a finding which indicates that the alpha adrenergic receptors may differ in these two tissues.

6. Further investigations on the pharmacology of WR 149,024.

a. Initial pharmacological actions:

Studies were carried out to characterize further the important cardiovascular actions of WR 149,024. Intravenous injections of 6.25, 12.5 or 25 mg/kg of this compound into intact anesthetized dogs and cats produced an immediate hypotension and bradycardia. These effects were not dose dependent and were independent of the parasympathetic nervous system, since they were also present in atropinized bilaterally vagotomized animals. This drug also induced an immediate 10-15 second period of apnea followed by rapid, shallow breathing for 10 minutes.

b. Ascending aortic blood flow:

WR 149,024, at an i.v. dose of 10 mg/kg, caused a slight increase in cardiac output, a simultaneous decrease in blood pressure and heart rate and a depression in the cardiac contractile force. Furthermore, ascending aortic blood flow did not decrease with the administration of WR 149,024 despite the reduction in contractile force and heart rate. These latter effects do not appear to be due to a direct myocardial action since the drug had little effect on force of contraction and heart rate in the isolated dog heart preparation. It therefore seems likely that the initial hypotension was due to a decrease in total peripheral vascular resistence.

c. Responses to catecholamines and angiotensin:

Normal vasopressor responses to epinephrine and norepinephrine were reversed or attenuated following WR 149,024. However, the vasopressor responses to angiotensin and the vasodepressor

responses to isoproterenol were not affected by the drug. Since this agent did not potentiate the isoproterenol depressor responses, it is unlikely that its action is to enhance beta adrenoceptor responses. Likewise, the lack of WR 149,024 antagonism of the pressor effects of angiotensin tends to rule out a non-specific interference with vasoconstriction.

The above data are consistent with the hypothesis that WR 149,024 is a specific alpha adrenergic blocking agent.

d. Endotoxin shock:

Experiments were carried out to determine the ability of WR 149,024 to protect dogs from a lethal dose of E. coli endotoxin administered intravenously. The specific objectives were: to determine the survival rate in dogs pretreated with WR 149,024 compared to controls; to determine the effect of WR 149,024 on the time course of response to endotoxin shock; and to observe the effects of the drug on the cardiovascular changes normally associated with endotoxin shock. A total of 20 dogs were treated with E. coli endotoxin, 1 mg/kg. Ten were pretreated with WR 149,024, 10 mg/kg; the other ten dogs were not pretreated and served as controls. All dogs in the control group died, whereas all dogs in the drug treated group survived and appeared normal after a 2 week period of observation. A fall in blood pressure was observed in the drug treated animals, but it was neither as rapid in onset nor as marked as the precipitous hypotension present in the control animals. Likewise, the heart rate was relatively unaffected in the treated group, while the control group had a sharp rise in heart rate corresponding to the sudden fall in blood pressure. In the control dogs, the fecal material was watery and contained mucus and blood, whereas the stools in the treated group remained essentially normal during the 3 hour observation period. The mechanism by which WR 149,024 was effective in preventing mortality in E. coli-induced endotoxin shock appears related to the early release of histamine, thus depleting the store of histamine which would otherwise be available to trigger this form of shock. In addition, no change in circulating levels of cortisol and catecholamines after endotoxin was seen.

The beneficial effects of WR 149,024 in endotoxin shock are most encouraging.

e. Blood glucose levels:

To investigate the effects of WR 149,024 on blood glucose levels, a dose of 10 mg/kg of body weight was administered via an intravenous catheter. The results showed an increase in plasma glucose of 10 to 20% over controls after administration of the drug.

7. Beneficial effects of WR 149,024 in hemorrhagic shock.

a. Background:

Hemorrhagic shock has been treated with, among other drugs, vasopressor agents and vasodilator agents. Phenoxybenzamine, an alpha adrenergic antagonist, has been used with some success. We have shown that WR 149,024 is a specific alpha adrenergic blocking agent with a selective effect on the cardiovascular system and no effect on intestinal muscle. Since we have shown this drug to be effective against endotoxin shock, it was natural to delineate its effectiveness against hemorrhagic shock.

b. Methods:

Thirty-two adult beagle dogs and 23 adult rhesus monkeys were studied. They were anesthetized with sodium pentobarbital and appropriate blood vessels were catheterized for i.v. infusion, blood sampling and the measurement of arterial blood pressure, EKG and heart rate. Cardiac output and blood volume studies were performed in some of the dogs. The animals were hemorrhaged into open glass reservoirs and maintained at 50 mm Hg aortic pressure for either 3 hours (dogs) or 3.5 hours (monkeys). At the end of this time period the blood was reinfused over 30 minutes. During reinfusion either WR 149,024, 10 mg/kg, or saline was given intravenously.

c. Results:

There was little difference between the treated and control groups within each species in arterial and venous blood pressure, heart rate, hematocrit, total plasma protein concentration, and red cell volume during the period of hemorrhagic hypotension or following blood infusion. However, an improvement in cardiac output, a reduced total peripheral resistance and an increase in whole blood and plasma volumes were evident in the drug treated group when compared to the appropriate control group following the return of shed blood. The most striking difference between these experimental groups was the survival record. Thirteen of the 16 dogs and all of the eight monkeys treated with WR 149,024 survived the 72 hour observation period while only three of the 16 control dogs and only five of the 15 control monkeys lived that long post hemorrhage. These results indicate that WR 149,024 may be efficacious in the treatment of hemorrhagic hypotension.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 192 Pharmacological effects of WR 2823 and other candidate anti-hypotensive drugs

Literature Cited.

References:

1. Demaree, G. E., Brockenton, R. E., Heiffer, M. H. and Rothe, W. E.: Antagonism of norepinephrine responses of isolated rabbit aorta by aliphatic sulfur-containing amines. J. Pharm. Sci. 60: 1743-1745, 1971.

2. Furchgott, R. F.: "Methods in Medical Research," A.D. Bruner, Ed., Yearbook Publishers, Chicago, Ill., 177-186, 1960.

3. Steel, R. G. D. and Torrie, J. H.: "Principles and Procedures of Statistics," McGraw-Hill Co., Inc., New York, N.Y., 109-110, 1060.

4. Herrington, K. A., Small, C. T., Meister, A. and Friedman, O. M.: Studies on latent derivatives of aminoethanethiols as potentially selective cytoprotectants, IV. enzymatic hydrolysis of cysteamine-S-phosphate. Cancer Res., 27: 148-151, 1967.

5. Caldwell, R. W. and Demaree, G. E.: The treatment of hemorrhagic shock with WR-149,024 (1,18-diamino-6,13-diaza-9,10-dithiaoctadecane tetrahydrochloride). Trauma (In press).

Publications:

1. Caldwell, R. W., Hutchinson, A. E., Heiffer, M. H. and Demaree, G. E.: Dissociation of alpha adrenergic blockade in gut and vasculature seen with two sulfur-containing aliphatic amines. Fifth Inter. Congr. Pharmacol. 14: 102, 1972.

2. Caldwell, R., Vick, J. and Heiffer, M.: Modification of endotoxin shock with 1,18-diamino-6,13-diaza-9,10-dithiaoctadecane (WR-149,024). Fed. Proc. 32: 782, 1973.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA OB 6522	73 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
	A. New	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	193
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede with Security Classification Code)*

(U) Synthetic Liposomes and Macrophage Activity

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

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13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
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FISCAL YEAR PRECEDING 73	1	12
FISCAL YEAR CURRENT 74	1	12

19. RESPONSIBLE DOD ORGANIZATION

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PII Redacted

ASSOCIATE INVESTIGATORS

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NAME: DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Procede EACH with Security Classification Code)

(U) Liposome; (U)Macrophage; (U)Virus; (U)Immunology; (U)Red Blood Cell

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Procede text of each with Security Classification Code.)

23(U) The liposome which is derived from lipid constituents of red cell membranes has many applications; liposomes offer a simple, reliable and convenient model for red cell membranes and can be used as an antigen of known lipid components. Studies on roles of liposomes in various immunologic events will provide new information on immunology and pathogenesis of infectious diseases which are current or potential problems in the Armed Forces.

24(U) Various biochemical extraction and assay methods, radioactive counting procedures.

25(U) 72 07 73 06 A study to determine the roles of lipid components of red cell membranes in the attachment of a radio-labelled Group A Arbovirus and Sindbis virus has been carried out. Lipid components of cell membranes of erythrocytes derived from various animal species including man have been arranged into multi-laminar membranes (liposomes). The conditions of virus attachment to the liposome were evaluated and found to be correlated well with those of the whole intact red cell from which the liposome is derived. The specific roles of individual lipid constituents of red cell membranes are currently being studied. The roles of antibody and serum complement on the uptake of sheep red cell-derived liposomes by cultured mouse macrophages have been investigated. The phagocytosis study has indicated that while antibody itself stimulates some uptake of liposomes, the presence of complement protein on liposomal surface significantly enhances the uptake beyond that of antibody alone. For technical reports see Walter Reed Army Institute of Research Annual Report, 1 Jul 72 - 30 June 73.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 193 Synthetic Liposomes and Macrophage Activity

Investigators.
Principal: MAJ John Mooney, MC.
Associates: CPT Joel Dalrymple, MC; MAJ Carl Alving, MC.

A. The Binding of Radiolabelled Sindbis Virus to the Red Blood Cell-Derived Liposomes

Background

The phenomenon of virus attachment to red blood cells is the basis of the hemagglutination test employed throughout virology, yet the mechanism of attachment remains unclarified.

In 1960, Salminen and colleagues noted that the total lipid fraction of human serum could inhibit the HA tests for tick-borne encephalitis virus (TEV) using avian RBC as target cells (1). Again, Salminen and his associates employed the acetone-extracted antigen to stimulate hemagglutination. Separation of the human serum lipids by column chromatography showed that the fractions containing both free fatty acids (especially the C-18 group) and free cholesterol inhibited the HA test. Further studies demonstrated that the choline-containing lipids such as lecithin and sphinogomyelin would also inhibit the HA test in the presence of cholesterol. Cardiolipin (a negatively charged lipid) also inhibited the HA assay. The methyl esters of the fatty acids were not capable of acting with cholesterol to inhibit the HA.

In 1962, Salminen showed that the hemagglutination of avian RBC by Sindbis virus antigen (a Group A Arbovirus) could be inhibited by aequous mixtures of lecithin and cholesterol or palmetic acid and cholesterol (2).

In 1967, Petkau and Chelock reported the penetration of thin films prepared from brain lipids by tobacco mosaic virus (TMV), a rod-like virus (3). They found that TMV penetration was Mg++ dependent and was enhanced if an electric field was applied across the thin lipid films. In a subsequent study, Petkau and Parker showed that the spherical virus Coxsackie B2 would not pass through the same membrane unless the membrane was modified by adding a protein obtained from the thioglycollate culture of Aerobacter cloacae (4). In the presence of this lipoprotein mixture, the passage of Coxsackie B2 virus required the presence of both Mg++ and the presence of an electric potential across the lipoprotein membrane.

Approach to the Problem

In our experiments we show: (1) intact virus can bind directly to artificial membranes made exclusively of lipids derived from red blood cell membranes, and (2) the characteristics of attachment to red cell lipids parallel those seen with the intact red cells from which the lipids are derived.

To prepare cell membrane lipids,we used unnucleated human and sheep erythrocytes because this laboratory has had difficulty obtaining satisfactory chloroform extracts from nucleated cells. Sheep red blood cells were selected for two reasons: (1) a rather large body of information has been obtained about the lipid composition of sheep erythrocytes; (2) sheep red cell-derived liposomes have been used in several immunologic studies. To study virus attachment to the membranes, we selected Sindbis virus, a Group A Arbovirus, for the following reasons: (1) it grows well in tissue culture and can be radiolabelled; (2) it is harmless to adults, yet it is closely related to the Eastern, Western and Venezualen encephalitis viruses which are of military significance.

All red cell collections were made into Alsiever's solution and stored at 4°C, until use in hemagglutination assays or lipid extraction. The hemagglutination assays were done in microtiter plates after the manner of Clarke and Casals (5), and read after incubation at 37°C for two hours.

The red cell membrane lipids were studied in the form of aequous dispersions which have a multilaminar crystalline structure similar to a set of concentric spheres. These crystals, called liposomes, were prepared in borate-phosphate-saline buffers after the manner of Haxby et al (6) from the chloroform-soluble lipids (i.e. sterols, phospholipids and glycolipids) of red cell ghosts lysed in low-osmotic strength buffer. The chloroform extracts lysed in low-osmotic strength buffer. The chloroform extracts were stored at -40°C in a manner of Dalrymple et al (7) and stored in borate-phosphate-saline buffer of pH 6.0 at -70°C until use.

The anti-Sindbis neutralizing antibody was raised in mouse ascitic fluid after the manner of Russel et al (8).

Virus in borate-phosphate-saline, pH 6.0, and liposomes in a BPS solution of the same or different pH were incubated together in glass tubes in a total volume of 800 lambda at 37°C for 45 minutes unless otherwise noted. In each case the BPS buffers used to swell

the liposomes were of the same pH and composition as those buffers used to determine the pH of the virus-liposome mixture.

Following the incubation period, the liposome-virus mixture was layered over a solution of 0.308 M sucrose in a 5 ml cellulose nitrate tube also containing a 70% (w/v) sucrose cushion of 500 lambda. The centrifuge tubes were spun in a Beckman SW 50.1 rotor at 50,000 rpm for 45 minutes at 4°C. Sindbis virus is easily brought intact through such a sucrose solution by this gravitational field (Dalrymple, personal observation). Following centrifugation, the liposomes were located at the air-water interface at the top of the tube and unattached virus was brought to the cushion. The centrifuge tubes were frozen with liquid nitrogen and cut into five consecutive with a jeweler's hacksaw. The segments were immediately placed into 16 x 100 mm glass tubes and allowed to melt. The fluid volume was measured and 200 lambda aliquants were dispensed into scintillation vials to which was then added a scintillation medium containing NCS solubilizer and liquiflour. Samples were counted for tritium for 10 minutes each at 4°C.

Results of the virus-liposome interaction are reported as the percentage of virus bound to the liposomes. The amount of virus present in each segment of the tube was standardized by multiplying the counts located in each segment by segment volume in lambda/1000 lambda. For example, a segment containing 900 lambda of fluid and 100 cpm/200 lambda was corrected to contain 900/1000 x 1000 cpm/200 lambda.

Results and Discussion

I. Attachment to Sheep Erythrocytes and Sheep RBC-Derived Liposomes

The hemagglutination study described in Table I shows that the virus agglutinates sheep RBC and this agglutination reaches a maximum at pH 5.8-6.0. The titer and pH sensitivity are identical to those for goose erythrocytes, the cell type ordinarily used for arbovirus hemagglutination studies.

The pH specificity of attachment to sheep erythrocyte-derived liposomes was studied using two buffer systems (Table II). The virus-liposome interaction shows a pH sensitivity similar to that seen in the interaction between the virus and the intact red cell. Red cells lyse in buffers whose pH is below 5.8. However, when the liposomes are swollen in and incubated with virus in buffers whose pH is below 5.8, the liposomes bind the virus as avidly as other sheep RBC-derived liposomes incubated at pH 5.8-6.0. The attachment process is not inhibited by incubation at low temperatures (Table III). Salminen (9)

has shown that the attachment of Group A Arbovirus to avian RBC is not inhibited by incubation at 4°C.

Conclusion

(1) Intact Sindbis virus (SV) binds to sheep, human, and avian erythrocytes.

(2) SV binds to liposomes prepared from lipids isolated from human and sheep erythrocyte membranes.

(3) The pH specificity of attachment to liposomes parallels the pH specificity observed with the hemagglutination of intact red cells.

(4) Neutralizing anti-Sindbis antibody blocks the attachment of SV to liposomes.

(5) The attachment of SV to liposomes is not affected by incubation at low temperatures.

We are presently examining the effects of charged and uncharged lipids on the attachment of Sindbis virus to human and sheep liposomes. We are also in the process of examining the structural lipids of the red cell membrane to determine if a particular lipid can modulate the electrostatic attachment of virus to the red cell membrane. This last effort is done in collaboration with Dr. Kenneth Joseph, a lipid chemist, presently working at WRAIR.

TABLE I

The hemagglutination of goose, sheep and human erythrocytes by Sindbis virus. Microtiter plates read after 2 hours at 37°C in the presence of borate-phosphate buffers of various pH. Each individual titration done in duplicate. In the first wells, the original viral material has a final dilution of 1:20.

SOURCE OF RBC	BLOOD GROUP	pH	TITER $^{-1}$
Goose		5.8	640
"		6.0	640
"		6.2	160-320
"		6.4	80
"		6.8	0
"		7.2	0
Sheep		5.8	640
"		6.0	640
"		6.2	160-320
"		6.4	80
"		6.8	0
"		7.2	0
Mooney	0+	5.8	160
"		6.0	80
"		6.2	0
"		6.4	0
"		6.8	0
"		7.2	0
Dalrymple	A+	5.8	160
"		6.0	80
"		6.2	0
"		6.4	0
"		6.8	0
"		7.2	0
Alving	AB+	5.8	160
"		6.0	80
"		6.2	0
"		6.4	0
"		6.8	0
"		7.2	0

TABLE II

The influence of pH on the attachment of Sindbis virus to sheep red cell-derived liposomes. The buffer used to swell the liposomes was the same buffer in which the virus and the liposomes were incubated together.

Buffer	Counts added per micromole liposomal phospholipid	pH	% Virus bound
Barbital-Acetate-HCl	14,000	4.7	88
		5.1	84
		5.4	90
		5.5	89
		5.7	85
		6.0	87
Borate-phosphate-saline	11.500	5.8	92
		6.0	90
		6.2	89
		6.4	73
		6.6	46
		7.0	25

TABLE III

Attachment to sheep erythrocyte-derived liposomes at different temperatures. Each lambda of Sindbis virus contains 7000 tritium counts. 1.0 micromole liposomal phospholipid is present in each incubation tube. All incubations performed in borate-phosphate-saline, pH 6.1 for 45 minutes. All liposome preparations were swollen at room temperature.

Experiment #	Incubation Temperature	Lambda Virus	% Binding	Mean % Binding
1	37°C	100	93	
		200	70	
		300	87	83
2	37°C	100	91	
		200	78	
		300	76	82
3	4°C	100	89	
		200	86	
		300	71	82

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TABLE IV

The attachment of Sindbis virus to sheep-derived liposomes following exposure of the virus to anti-Sindbis antibody. Ascitic fluid collections from both mice immunized to Sindbis and unimmunized mice are diluted in borate-phosphate-saline buffer, pH 6.0, and incubated with the virus for 20 minutes at 37°C. The ascitic fluid/buffer mixtures are then added to liposomes and incubated for 45 minutes at 37°C. Each incubation mixture contains approximately 11,000 tritium counts per micromole liposomal phospholipid. The % binding of the last entry reflects the amount of label remaining in the sectors of the bue above the cushion.

Ascitic Fluid	Ascitic Fluid Dilution	% Binding
None	-	80
Unimmunized mice	1:10	76
Immunized mice	1:1000	73
Immunized mice	1:100	46
Immunized mice	1:10	3
Immunized mice (liposome-free control)	1:10	1

TABLE V

The effects of pH on the attachment of Sindbis virus to liposomes from human 0+ erythrocytes. The borate-phosphate-saline buffer used to swell the liposomes was the same buffer used to incubate the virus and liposomes together.

Donor	Tritium counts/micromole liposomal phospholipid	pH	% Binding
Hoskins	10,500	6.0	77
		6.3	38
		6.6	32
		7.1	12
Mooney	14,000	5.8	93
		6.0	81
		6.2	60
		6.4	57
		6.6	39
		7.0	17

TABLE VIa

Binding to a liposome preparation from donor Mooney where 100 lambda of liposomes (1 micromole liposomal phospholipid) are serially diluted in borate-phosphate-saline, pH 6.2, and then incubated with approximately 14,000 counts of tritiated Sindbis virus per tube.

DILUTION	% BINDING
1:1	52
1:10	48
1:20	36
1:40	32
1:80	27
1:160	28

TABLE VIb

Binding to two sheep preparations from the same donor sheep where 100 lambda of liposomes (1 micromole liposomal phospholipid) are serially diluted in borate-phosphate-saline, pH 6.2, and then incubated with approximately 14,000 counts of tritiated virus preparation per tube.

DILUTION	# SAMPLES	MEAN % BINDING
1:1	2	76
1:10	1	63
1:20	2	55
1:40	2	44
1:80	2	33
1:160	2	25
1:320	1	18

B. The Phagocytosis of Antigen-Bearing Liposomes by Mouse Macrophages

Background

Using the procedures described by Haxby et al (10), the lipids of sheep erythrocyte membranes, including Forssman antigen (a membrane glycolipid), are employed to make multilaminar crystals in aequous media called liposomes. These lipid crystals dispersed in aequous solution have Forssman antigen incorporated into their structure (10) and it is, therefore, possible to study the roles of antibody and complement on the phagocytosis of these liposomes. Alving and Kinsky (11,12) have shown that anti-Forssman antibody will bind to liposomes and the presence of antibody on the liposomes will activate serum complement, causing the complement proteins to bind and secondarily disrupt the integrity of the liposomal membrane in a manner analogous to red cell lysis by antibody and complement.

We utilize red cell-derived liposomes because: (1) they are relatively simple, being constructed solely out of lipid, unlike the cell membrane which is a complex of lipids and proteins; (2) we can directly measure and to a certain extent control the amounts of antibody and complement proteins bound to the liposome surfaces; (3) unlike red cells, liposomes do not lyse when participating in a complement reaction; (4) the liposomes not phagocytized by macrophages can be harvested with simple procedures.

Approach to the Problem

Liposomes are prepared after the manner of Haxby et al (10) with the following lipid constituents: sphingomyelin (extracted from beef brain), cholesterol, dicetylphosphate (two C-16 fatty acids esterified to a phosphate molecule, and having a net negative charge), and sheep red blood cell lipids. The molar proportions of these ingredients is SM/CHOL/DCP/SEL = 1/1.5/0.22/1. This mixture mimics the cholesterol/phospholipid ratio usually found in mammalian RBC membranes (approximately 0.75) (13).

The antibody source used is identical to that of Alving and Kinsky (11,12) namely rabbit anti-sheep antibody supplied diluted 1:2 in glycerin. The amounts of antibody given here are amounts of undiluted rabbit serum. The DIFCO preparation was extensively dialysed against saline at 4°C and then heated to 56°C for 30 minutes to eliminate the presence of any active complement protein. This material was then clarified by centrifugation at 37,000 x g for 15 minutes at 4°C. The dialysed, heated clarified antibody preparations were then stored at -70°C until use. Methylated bovine serum albumin was obtained commercially and was diluted into saline containing physiologic amounts of calcium and magnesium ions. This proteins, here abbreviated m-BSA, has a strong positive charge. Guinea pig serum was obtained by decapitation and was stored at -70°C until use. Except for the dialysis of the rabbit anti-sheep serum preparation (RAS), all saline used contained physiologic quantities of calcium and magnesium ions (SAL++).

Cultures of peritoneal macrophages are harvested from normal mice after the manner of Cohn and Benson (14). The cells are grown in 30 mm diameter Falcon plastic dishes overnight in 20% aged guinea pig serum and Medium 199 containing penicillin and streptomycin in an atmosphere of 10% CO_2. After the overnight incubation, the cells are washed,once with Medium 199 and 1.25 ml of fresh Medium 199,without additional serum or protein added back to the washed macrophage monolayers. This small portion of Medium 199 contained penicillin and streptomycin. Counting experiments employed a Coulter counter (70 micron aperature window) revealed a typical dish to contain 1.0-$2x10^6$ macrophages following overnight incubation and washing. We found it necessary to exclude serum from the experimental cultures because the presence of even modest quantities of protein can retard the sedimentation of liposomes to which antibody alone was bound (LA).

The liposomes are prepared on the day of the experiment and exposed to varying quantities of antibody, m-BSA, and serum complement at room temperature for 30 minutes. The liposomes (LA, LAC', L-mBSA) are washed twice in SAL++ and added in small quantities to the culture dishes. Each dish received 8millimicromole of liposomal phosphate, unless otherwise noted. The liposomes from four such dishes were collected and pooled at each given point in time. This procedure allowed the dilution of the liposomes in such a manner as to completely retard the clumping of liposomes, especially LA preparations. The cultures are placed in a 37°C incubator in an atmosphere of 10% CO_2. The culture dishes remain stationary throughout the experiment and sample dishes are removed at various times to harvest the liposomes. When dishes were placed on shaking platforms at various speeds, it was found that they tended to clump, especially, again, the LA preparations.

During the harvesting procedure, the medium was removed and the monolayers washed twice with SAL++. The media and washings were collected into Corex tubes and 40 lambda of RAS added to each tube. After an incubation period of 10 minutes at room temperature, the tubes were spun at 37,000 xg for 15 minutes at 4°C to collect the liposomes. The pellet was washed once in a large volume of SAL++ to remove soluble phosphate. Each pellet of liposomes was resuspended in 500 lambda SAL++ and 160 lambda aliquots taken for phosphate assay. It should be pointed out here that these rapid centrifugations will destroy the integrity of macrophages, but to further rule out the pellet phosphate as coming from the culture cells, each experiment had cells-only controls run at zero and 24 hours, and the phosphate measurable after centrifugations and washings was always negligible.

The amount of phagocytosis in each individual experiment is measured by comparing the amount of liposomal phosphate obtained at time zero with that still remaining in the medium after the passage of different time periods. Morphologic observations were made on unfixed monolayers both before and after each liposome harvest at 100 x magnification. The liposomes were visible at this magnification and in any one dish, the liposomes were observed to be (10)in suspension in the medium, (11)adhering to macrophages, or (12)inside the macrophages.

Results and Discussion

I. The resistance of SM/CHOL/DCP/SEL to destruction by lysosomal enzymes.

Cohn and Wiener (15)reported that macrophage cultures contain large quantities of lysosomal enzymes released into the medium by the cells. Table I shows that the exposure of liposomes to 36 hour-old macrophage culture medium did not reduce the amount of liposomal phosphate recovered 24 hours later. This experiment also verifies the morphologic observations that these liposomes did not stick to the plastic dish surface.

II. The uptake of liposomes pre-exposed to normal rabbit serum.

The mean uptake of liposomes exposed to 40 lambda of heated, dialysed normal rabbit serum per micromole liposomal phosphate is presented in Table II. The mean uptakes are 3% at 10 hours and 16% at 24 hours.

III. The uptake of liposomes alone by macrophages.

Macrophages will phagocytize the liposomes in the absence of antibody or complement on the surface of the liposome (Table III).

IV. The amounts of protein bound to liposomes under various conditions.

Table IV indicates that the incubation of liposomes with antibody in the presence of fresh guinea pig serum causes the addition of a very large quantity of protein to the liposome membranes. It was also observed that the amount of m-BSA bound to the liposomes was approximately equal to that of 40 lambda RAS. The data of Table IV involving the binding of antibody or antibody and complement reconfirms the observations originally made by Alving and Kinsky (11).

V. The influence of antibody along on the phagocytosis of liposomes.

Table V demonstrates that the presence of antibody can enhance the uptake of liposomes over the phagocytosis seen in the absence of antibody, as measured by uptake of either the liposomes themselves, or the uptake of liposomes pre-exposed to normal rabbit serum. As the amount of antibody is increased, the amount of protein bound by the liposomes is increased and the phagocytosis is enhanced. At the end of 34 hours, the amount of phagocytosis begins to approach 100%.

VI. The influence of complement on the uptake of liposomes (LAC').

The data in Table VI suggests that the phagocytosis of LAC' is strikingly enhanced by complement. However, this effect is not clear because the complement was not absorbed with sheep erythrocytes at 4°C. Absorption with sheep erythrocytes removes anti-sheep RBC antibodies present in the guinea pig serum while preserving the C' if done at low temperatures. These experiments will have to be repeated using absorbed guinea pig serum.

VII. The phagocytosis of liposomes treated with methylated bovine serum albumin.

Table VII demonstrates the remarkable uptake of liposomes with membrane bound m-BSA. This protein has a strong positive charge due to the large number of methyl groups and it is highly attracted to the membrane surface whose charge is negative because of the presence of DCP (and probably phosphatidylserine from the sheep erythrocyte lipids). We have discovered a new opsonin in methylated BSA. Most biological membranes have a net negative charge at physiologic pH, and could, therefore, bind this protein.

Examination of the data in Tables IV and VI raises the criticism that the antibody effects in the presence or absence of complement could simply be a weight phenomenon--the more protein bound, the heavier the particle, the greater the phagocytosis. This criticism is refuted by the m-BSA experiments where liposomes with m-BSA were phagocytized more than twice as fast as liposomes with 40 lambda RAS, yet both have comparable amounts of protein bound to the liposomal membrane. Furthermore, liposomes pretreated with 200 micrograms of m-BSA per micromole phosphate are phagocytized as efficiently as those liposomes exposed to antibody and unabsorbed guinea pig serum having 13 times more protein bound to the liposome membranes.

Conclusion

(1) A system has been described for the phagocytosis of a lipid membrane formed from the lipids of red cell ghosts and containing Forssman antigen, a glycolipid.

(2) Anti-sheep red cell antibody binds to liposomes containing sheep red cell lipids and enhances their uptake in proportion to the amount of antibody bound to the surface of the liposome.

(3) Methylated bovine serum albumin, a positively-charged protein, binds to the negatively charged liposome and facilitiates a striking uptake of these membranes in the absence of the classical opsonins antibody and complement.

(4) The uptake of the liposomes is not a strict function of the amount of protein bound.

(5) This project will shortly be completed by a proper study of the uptake of liposomes treated with both antibody and complement, using adsorbed guinea pig serum as a source of complement activity.

TABLE I

Exposure of liposomes to macrophage tissue culture medium in which the cells had grown for 36 hours. The medium contained 20% aged commercial guinea pig serum. Each dish contained 0.32 micromole liposomal phospholipid. Each time interval reading represents the mean of two dishes. The liposomes were incubated with the medium in plastic dishes for a total of 24 hours at 37°C.

Time (Hrs.) of Harvest	Millimicromoles phosphate dish	% Change
0	299	-
6	278	-7.0
12	298	-0.3
24	289	-3.0

TABLE II

Exposure of one micromole of liposomal phospholipid to 40 lambda of dialysed normal rabbit serum heated to 56°C for 30 minutes. The liposomes are then added to dishes and collected at various times. The values below represent the mean of two experiments.

Harvest Time (hrs.)	% Phagocytosis
10	3
24	16

TABLE III

Washed liposomes are added to macrophage cultures and harvested at various times. The liposomes were not exposed to antibody before adding them to the dishes. The values given below are means of the harvests from two experiments. In each experiment, each harvest was done in duplicate.

Harvest Time (hrs.)	Millimicromoles Phosphate/dish	% Phagocytosis
0	184	-
4	162	12
10	167	9
24	142	23

TABLE IV

The uptake of liposomes pre-exposed to antibody and complement at room temperature for 30 minutes. The source of complement is unabsorbed guinea pig serum. The heated complement was prepared by heating in a water bath at 56°C for 30 minutes. The values given below are the mean % phagocytosis values derived from several experiments.

Conditions before exposure to cultures	Harvest Time (hrs.)	% Phagocytosis	# Experiments
40 lambda RAS+6ml C'	10	44	2
	24	55	4
180 lambda RAS+6ml C'	10	58	5
	24	78	5
40 lambda RAS+6ml heated C'	24	73	2

TABLE V

The phagocytosis of methylated bovine serum albumin (m-BSA) treated liposomes. The phagocytosis percentages below represent the mean of several experiments.

Micrograms m-BSA per micromole liposomal phospholipid	Harvest Time (hrs.)	% Phagocytosis	# Experiments
25	10	8	2
25	24	29	2
50	10	14	3
50	24	39	3
100	10	38	3
100	24	68	3
200	10	50	2
200	24	73	3

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 193 Synthetic Liposomes and Macrophage Activity

Literature Cited.

References:

1. Salminen, A., Renkonen, O.V., and Renkonen, O.: In: Biology of Viruses of the Tick-Borne Encephalitis Complex. Ed. H. Libikova; Acad. Press, 1962, p. 104-112.

2. Salminen, A.: Virology 16: 201, 1962.

3. Petkau, A. and Chelack,W.S.: Biochem. Biophys. Acta. 135: 812-824, 1967.

4. Petkau, A. and Parker, W.L.: Biochem. Biophys. Acta. 135: 825-830, 1967.

5. Clarke, D.H. and Casals, J.: Am. J. Trop. Med. & Hyg. 7: 561, 1958.

6. Haxby, J.A., Kinsky, C.B., Kinsky, S.: Proc. Nat. Acad. Sci. 61: 300-307, 1968.

7. Dalrymple, J.M., Teramoto, A.Y., Cardiff, R.D. and Russell, P.K.: J. Imm. 109: 426-433, 1972.

8. Russell, P.K., Chiewsilp, D. and Brandt, W.E.: J. Imm. 105: 838, 1970.

9. Salminen, A.: Ann. Med. Exp. Biol. Fenn. 37: 400, 1959.

10. Haxby, J., Kinsky, C.B., Kinsky, S.: Proc. Nat. Acad. Sci. 61: 300, 1968.

11. Alving, C.R., Kinsky, S., Haxby, J.A. and Kinsky, C.B.: Biochem. 8: 1582, 1969.

12. Alving, C.R. and Kinsky, S.: Immunochem. 8: 325, 1971.

13. Wessels, J.C. and Veerkamp, J.H.: Bioch. Biophys. Acta. 291: 190, 1973.

14. Cohn, Z.A. and Benson, B.: J. Exp. Med. 121: 153, 1965.

15. Cohn, Z.A. and Wiener, E.: J. Exp. Med. 118: 1009, 1963.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6523	73 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
	A. New	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	194
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Development of an Organ Culture Method from Intestinal Biopsies

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

00 26 00 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 01	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER: NA

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 73	1	10
FISCAL YEAR CURRENT 74	1	15

19. RESPONSIBLE DOD ORGANIZATION

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TELEPHONE: 202-576-2024

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

ASSOCIATE INVESTIGATORS

NAME: Richardson, CPT, W.

NAME: DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U)Organ Culture; (U)Intestine;(U)Phase Microscopy(U)Electron Microscopy

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23(U) To develop a reliable and reproducible method in which organ cultures of small and large intestines of experimental animals and man be cultured and maintained without producing alterations of function and metabolism. The cultured gut will be employed in studies of interactions between the mucosa and various microbes including viruses and microbe-derived toxins by various methods. These results will provide new information which should clarify the pathogenesis of acute diarrheal diseases in military personnel.

24(U) Conventional morphologic parameters including phase-contrast, light, electron microscopy and histochemistry and isotope tracer methods will be used. Methods such as interference microscopy and cinematography will be supplemented as the work progresses.

25(U) 72 07 73 06 A new organ culture chamber has been developed by a modification of Rose's tissue culture chamber. This chamber was specifically designed for easy observations of cultured gut by phase-contrast microscopy. In this chamber, the small and large intestinal tissues from both fetal mice and guinea pigs have been successfully cultured up to 5 days with no structural evidence of tissue alterations. Analyses of function and metabolisms of growing bowel tissues by immunotracer and biochemical methods have been initiated, while a system to record continuous growth of cellular elements and tissues of cultured gut by cinematography is being completed.
For technical reports see Walter Reed Army Institute of Research Annual Report, 1 Jul 72 - 30 June 73.

* Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 194 Development of an Organ Culture Method from Intestinal Biopsies

Investigators.
Principal: Akio Takeuchi, M. D.
Associate: CPT Walter Richardson, MC

Description

To develop a reliable and reproducible method for the organ culture of the small and large intestine of experimental animals and man. The cultured gut will be employed in: (1) Studies by various parameters of responsiveness of the gut mucosa and submucosa to various enteric microbes and microbe-derived toxins. Special attention will be paid to cinematographic recordings as well as conventional static observations on various cellular interaction of the gut mucosa with various microbes and toxins. (2) Studies of replication sites of enteroviruses.

These results should provide valuable new information which will clarify as yet unsolved problems in pathogenesis of acute infectious diarrheal diseases common in military personnel at home and overseas.

Background

Organ culture techniques have proven to be of considerable value in the study of respiratory tract infections in man and animals. Recently, interest has developed in the organ culture of gut tissues for the cultivation of enteric viruses. Rubenstein and Tyrrell (1971), Dolin and Stenhouse (1970), and Derbyshire and Collins (1971) provided evidence of the multiplication of viruses in organ cultures of the small intestine of the human embryo. Kagnoff et al (1972), in their organ culture study of adult rabbits, have reported that the metabolic function and synthesis of macromolecular substances including secretory IgA were still active after 24 hours culture in a Petri dish.

527-825 O - 74 - 11

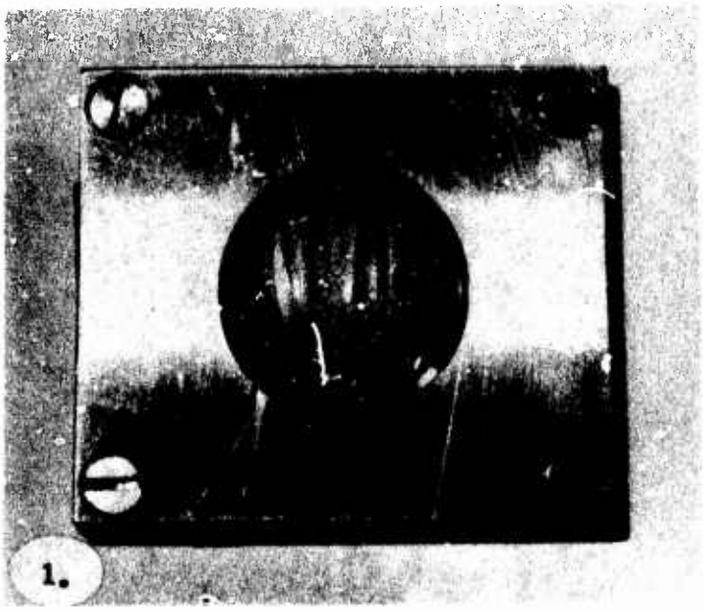
Most of the chambers used by the above investigators appeared adequate for the growth of certain viruses and for evaluation of limited metabolic activities in the cultured bowel. These chambers do not allow for an immediate and accurate method of visualization and biochemical analysis at any given time during the maintenance of the infected culture. This shortcoming prompted the development of a new chamber and a convenient observation system during actual organ culture. After careful review of appropriate literatures and advice from cell and tissue culture specialists, we have chosen a new organ culture chamber which meets the object of our studies by allowing direct morphologic observation on cells and tissues of the gut.

Progress

A new organ chamber and a unit containing phase contrast microscope have been designed and constructed with the assistance of Dr. Tokio Asaka, Director of Research, Cine-Science Laboratory, Tokyo, Japan, and the Division of Instrumentation, WRAIR. The chamber was developed by modifying Rose's chamber (Fig. 1), and provides easier handling of tissues and better visualization of growing cells and tissues under the microscope (Fig. 2). The unit is composed of a plastic housing containing phase-microscope and a thermocontrol device which regulate and maintain the inside temperature at 37°C. A time-lapse cinematography instrument can be attached to this unit as shown in Figure 3.

After numerous attempts to choose appropriate culture medium, we have found Medium 199 with 10% calf serum containing both streptomycin and penicillin satisfactory. However, the concentration of serum varied from 20% to 5% depending upon the age of the donor's fetus.

Contrary to the general belief that the constant supply of oxygen is imperative for maintaining an organ in culture, the fetal gut can grow without it for up to 48 hours and show no structural degeneration. With daily changes of medium, we have been able to maintain successfully fetal small and large intestine of mouse and guinea pigs up to 5 days (Fig 4 and 5). The direct visualization on the gut mucosa in the culture chamber has demonstrated occasional rhythmic movements of the muscle and villi up to 3 days. Analyses of function and metabolisms of growing bowel tissues by immunotracer and biochemical methods have been initiated, while a system to record continuous growth of cellular elements and tissues of cultured gut by cinematography is being completed.



A new culture chamber modified from Rose's chamber.



Chamber placed under phase contrast microscope.



Cinematographic equipment with phase contrast microscope.



Figures 4 and 5 - Fetal small intestinal mucosa of mouse in culture chamber (Fig. 4, 2 days; Fig. 5, 3 1/2 days culture). Growing intestinal villi show no evidence of structural alteration. A capillary (CAP) contains several red blood cells. X200.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 194 Development of an Organ Culture Method from Intestinal Biopsies

Literature Cited.

References:

1. Derbyshire, J.B. and Collins, A.P.: The multiplication of Talfan virus in pig intestinal organ cultures. Res. Vet. Sci. 12: 367, 1971.

2. Dolin, R. et al: Establishment of human fetal intestinal organ culture for growth of viruses. J. Inf. Dis. 122: 227, 1970.

3. Rubenstein, D., Milm, R.C., Buckland, R., and Tyrrell, D.A.J.: The growth of the virus of epidermic diarrhea of infant mice (EDIM) in organ culture of intestinal epithelium. Br. J. Exp. Path. 52: 422, 1971.

4. Kagnoff, M.T. et al: Organ culture of rabbit small intestine: Prolonged in vitro steady state protein synthesis and secretion and secretory IgA secretion. Gastroenterol. 63: 541, 1972.

Publications:

1. Takeuchi, A. and Asaka, T.: Cinematographic observations on intestinal villi in vivo: Introduction of a new experimental procedure. Fed. Proc. 32: 321, 1973.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6524	73 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	8a. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
	A. New	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	195
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede with Security Classification Code)

(U) Sheep Red Cell Model for G6-PD Deficiency

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

00 26 00 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

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c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 73	.5	4
FISCAL YEAR CURRENT 74	0.5	4

19. RESPONSIBLE DOD ORGANIZATION

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ASSOCIATE INVESTIGATORS

NAME: Giles, CPT, R.

NAME: Hildebrandt, LTC, P. DA

PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Procede EACH with Security Classification Code)

(U)G6-PD Deficiency; (U)Sheep; (U)Primaquine; (U)Malaria; (U)Model

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Procede text of each with Security Classification Code.)

23(U) To determine if sheep will develop a hemolytic crisis when exposed to anti-malarial drugs such as primaquine. Sheep red blood cells have been determined to be G6-PD deficient. Sheep may serve as a model for the pathogenesis of the hemolytic crisis which occurs in G6-PD deficiency in military personnel when exposed to such anti-malarial drugs as primaquine.

24(U) Conventional methods and the utilization of isotope tracers such as Fe-fifty-five and Fe-fifty-nine are utilized to monitor the hemogram following exposure to primaquine.

25(U) 72 07 73 06 Sheep hemograms were monitored by routine methods and isotope tracer techniques during baseline studies and post oral, IM and IP dosing of primaquine. No evidence of hemolysis was noted in any sheep given primaquine regardless of route. Studies will be initiated with the Department of Hematology to determine the mechanism which allows the sheep red blood cell to withstand hemolysis when exposed to primaquine. The current study has provided new information on ferrokinetics in sheep.
For technical reports see Walter Reed Army Institute of Research Annual Report, 1 Jul 72 - 30 June 73.

*Available to contractors upon originator's approval.

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Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 195 Sheep Red Cell Model for G6-PD Deficiency

Investigators.
Principal: LTC Paul K. Hildebrandt, VC
Associates: MAJ Charles A. Montgomery, VC; CPT Ralph C. Giles, VC

Background

The basis for this study was to determine if sheep may serve as a model for glucose-6-phosphate dehydrogenase deficiency and the hemolytic crisis which occurs with exposure to anti-malarial drugs such as primaquine.

Approach to the Problem

Six adult sheep were used in this study. Blood was collected for baseline hematology and blood was also collected for G-6-P-D assay. All sheep had G-6-P-D levels considered to be G-6-P-D deficient by human standards.

Sheep	International Units
31	.116
47	.047
55	.048
83	.032
84	.050
85	.050

Blood was also collected for serum iron determinants. All sheep were given 500 μCi Fe^{55} intravenously via the jugular vein. Blood samples (3-4 ml) were collected in heparinized syringes at 15 minutes, 1,2,3 and 4 hours post-isotope injection. Blood samples were collected twice weekly for the following 17 weeks and iron uptake curves plotted. Hematocrits, hemoglobulin red cell counts, reticulocytes and the presence of Heinz body formation were also examined.

Forty-five days post-Fe^{55} injection, Ferrous citrate (Fe^{59}) with a specific activity of 23 mCi/mg was diluted in a 0.1% sodium citrate solution to a concentration of 5 μCi/ml. Fifty μCi in a 10 ml solution was injected intravenously via the jugular vein. Blood samples were collected 15 minutes, 1,2,3, and 4 hours post-injection and also twice a week for the next 10 weeks for plotting a Fe^{59} uptake curve. These two iron isotopes were given 45 days apart in order to have an older red cell population and a young red cell population tagged with isotope.

All samples were counted in a Packard Tri-carb liquid scintillation spectrometer using two channels to count the Fe^{55} and Fe^{59}. Samples for Fe^{59} were also counted in a Nuclear Chicago Automatic Gamma Counter to verify the Fe^{59} samples counted in the Tri-carb. Both Fe^{59} curves were identical.

Sixty-nine days post-Fe^{55} injection and 24 days post-Fe^{59} injection, two sheep were given 45 mg primaquine orally for 10 days. Two sheep were given 45 mg orally and received a second 45 mg oral dose in 7 days. Two sheep served as controls.

The Fe^{55} and Fe^{59} curves and the hematological parameters examined did not vary from normal nor from the control animals during the following 10 days. Additional primaquine was given in the following manner. The two sheep which had received the 45 mg primaquine daily for 10 days were given 150 mg orally, daily, for six additional days, followed with 60 mg intramuscularly, daily, for six days. The two sheep which had received the two 45 mg oral doses a week apart received 60 mg intraperitoneally, daily, for six days.

Results and Discussion

At no time did the iron uptake curves or hematological parameters examined deviate from normal nor from the controls. It is apparent that sheep, although G-6-P-D deficient, will not develop hemolysis in the presence of primaquine.

Although sheep do not appear to be a model for G-6-P-D deficient studies associated with hemolytic crises, a most intriguing question arises. What metabolic pathway does the sheep red blood cell possess which allows it to withstand hemolysis in the presence of primaquine? Studies are currently being formulated to resolve this question.

PROJECT 3A061102B71Q
COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00
Communicable Diseases and Immunology

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA 0A 6440	73 07 01	DD-DR& %(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
72 07 01	D. CHANGE	U	U	NA	NL	☑ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A061102B71Q	00	165
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 114(F)			

11. TITLE (Precede with Security Classification Code)*

(U) Parasitic Diseases (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
54 09	CONT	DA	C. In-House

17. CONTRACT/GRANT NA

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:*

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 73	3	75
FISCAL YEAR CURRENT 74	6	210

19. RESPONSIBLE DOD ORGANIZATION

NAME:* Walter Reed Army Institute of Research

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20. PERFORMING ORGANIZATION

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Division of CD&I

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PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

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21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Parasite; (U) Schistosomiasis; (U) Pathology; (U) Primate; (U) Chemotherapy

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23(U) The purpose of this research is to study various physiological, immunological and ecological aspects of parasitic diseases of military importance toward the goal of gaining a better understanding of natural susceptibility, acquired resistance and effectiveness of therapeutic agents for the prevention, suppression and treatment of these infections.

24(U) Through careful perusal of pertinent literature and discussion with other scientists both classical and new methods are used to set up controlled experiments.

25(U) 72 07 - 73 06 Trypanosoma rhodesiense (Wellcome strain) were processed in a RIBI Cell Fractionator to obtain soluble and particulate fractions. Mice were inoculated with a soluble fraction, a particulate fraction and lyophilized parasites. Another immunizing inoculation was given 3 weeks later. Two weeks later these mice along with 10 healthy control mice were challenged with viable parasites. All controls died in an average of 4.2 days. From 50 to 100 percent of animals in immunized groups survived without detectable parasitemias. Mice receiving the particulate fraction of the parasite had the greatest degree of protection. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 July 72 - 30 June 73.

*Available to contractors upon originator's approval.

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Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 165 Parasitic diseases

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1. Immunization of cattle with gamma irradiated Trypanosoma rhodesiense.

Trypanosoma rhodesiense can be passaged experimentally through many different species of animals without losing its infectivity for man. When Heisch et al. experimentally transmitted T. rhodesiense from a naturally infected bush buck (Tragelaphus scriptus) to man they provided direct evidence that wild game animals are reservoirs of the parasites causing human sleeping sickness. Wilde and French showed that domestic cattle are susceptible to T. rhodesiense and postulated that these animals could also act as reservoir hosts for sleeping sickness. This was demonstrated during an epidemic of sleeping sickness in Western Kenya in 1963 when trypanosomes isolated from cattle produced an infection in man typical of that caused by T. rhodesiense.

Duxbury and Sadun reported that irradiated T. rhodesiense induced a strong resistance in laboratory rats and mice to a challenging infection with the same parasite. These observations were confirmed and extended by further investigations in rodents and primates.

Since domestic cattle may play an important role in the transmission of human sleeping sickness, experiments were undertaken to study trypanosomiasis in cattle. Particular emphasis was placed on the induction and persistence of immunity produced in bovines by inoculation with T. rhodesiense attenuated by exposure to gamma radiation.

Experimental Animals

Twenty one cattle of both sexes were used in 4 experiments. In a pilot experiment (Exp. I) Hereford cattle were used. Animals in Experiments II, III and IV were mainly of a Boran breed weighing between 400 and 700 pounds. The cattle were provided by the Veterinary Department, Kabete, Kenya and were maintained at the Trypanosomiasis Research Laboratory in Kabete. Laboratory rats were used as a source of immunizing and challenging inocula and mice were subinoculated with blood from the cattle to detect subpatent parasitemias.

Parasites

A monomorphic strain of T. rhodesiense (Wellcome) which had been maintained in laboratory rats for many years and a relatively recent isolate of T. rhodesiense (EATRO #1886) were used in these studies.

Parasites for immunization and challenge were obtained from infected laboratory rats. The rats were anesthetized with ether and bled by cardiac puncture with a syringe containing heparin. In the first experiment whole infected rat blood was irradiated and inoculated into the cattle. In experiments II and III infected blood was centrifuged for 10 minutes at 1000 G and the plasma discarded. The thick buffy coat was then transferred to a 0.01M phosphate buffered saline solution (pH 7.8) containing 5% (w/v) glucose and 10% (w/v) fetal bovine serum. The buffy coat was washed twice at 1000 G and reconstituted with the buffer. The number of trypanosomes in the resulting suspension was determined by diluting an aliquot of the sample in a red blood cell pipette with a 0.05% Nile blue sulfate solution. After 15 minutes the pipette's contents were mixed, expressed into a hemacytometer and the trypanosomes counted. Immunizing inocula were irradiated in a Gammacell cobalt 60 irradiator at approximately 70 kilorads. Immunizing and challenging doses of T. rhodesiense were injected into the jugular veins of the cattle. Each immunizing inoculum of irradiated trypanosomes was also injected into rats to determine whether the parasites had been rendered noninfective by their exposure to irradiation.

Patent infections in the cattle were detected by examining approximately 200 oil immersion fields on Giemsa stained thick blood films obtained from capillary blood of the tail. To detect subpatent infections, 0.5 ml aliquots of blood from the jugular vein were inoculated intraperitoneally into mice. Rectal temperatures were done 6 days per week while packed cell volumes and leucocyte counts were done weekly.

All cattle were tested and proven negative for subclinical trypanosomiasis by both blood films and subinoculation before their inclusion in the study.

Experiment I

Three Hereford cattle were given three injections of irradiated whole rat blood containing T. rhodesiense. Three x 10^9 irradiated trypanosomes were inoculated in 3 equal doses given one week apart. One week after the third injection, the three experimental animals and 3 untreated controls were each challenged with 1 x 10^5 viable trypanosomes. The three controls became patent on the third day after challenge and two experimental animals became patent on days 6 and 13. The third experimental animal did not develop a patent infection. No parasites could be found in this animal by blood smear examination or by subinoculating its blood into mice. When the animal was rechallenged with 1 x 10^5 viable T. rhodesiense 5 months after the first challenge, a patent infection developed.

Table 1

Number of Irradiated Trypanosomes ($x10^9$) in Immunizing Inoculations

Experiment No.	Animal No.	Day of Immunization 0	4-5	7	11	13	15-16
II	6359	18.4	9.0	8.0	5.4	10.1	7.8
	6360	22.9	9.0	8.0	5.4	10.9	7.8
III	6340	4.6	11.0	6.0	5.7	6.1	4.4
	6351	4.6	11.0	6.0	5.7	4.6	4.4
	6358	4.6	11.0	6.0	5.7	6.1	4.4

Experiment II and III

These experiments were conducted in order to determine whether a stronger degree of immunity could be obtained with larger numbers of irradiated trypanosomes relatively free from contaminating rat host materials (Table 1). Stained smears of the immunizing suspensions contained only a few erythrocytes and leucocytes although thrombocytes were abundant. In both experiments the injections were well tolerated by the animals and no evidence of anaphylaxis was observed. Packed cell volumes, leucocyte counts and body temperatures showed no abnormal variation during the period of immunization. One week after the last immunizing injection the animals were challenged with approximately 1×10^4 viable T. rhodesiense. Although the control animals in both experiments developed patent infections, no parasites were seen in the blood of the cattle which had previously received irradiated parasites. Blood from the immunized cattle did not infect mice, but blood from the control animals produced infections in mice for 8 to 10 weeks after challenge (Table 2). Although the packed cell volumes did not show abnormal variation in either immunized or control animals after challenge, a leucopenia developed in the controls during the onset of patent parasitemia but usually subsided within a week. Fevers were detected in all the controls at the time trypanosomes first appeared in the blood and in some of them at the time parasites reappeared in large numbers.

Rats were given single injections of irradiated trypanosomes from the same aliquots as those used for cattle. None of the rats became infected. When these animals were subsequently challenged with unirradiated trypanosomes, all the control rats died of the infection, but 76% of the rats that had received a single immunizing inoculation were protected against challenge (Table 3).

All experimental and control cattle of Experiments II and III were rechallenged approximately 8 months after the initial challenge. Three additional animals which had never been infected with T. rhodesense were also challenged at the same time for use as controls. The immunized animals again did not develop patent infections and their blood did not infect mice after subinoculation up to 3 months after challenge. The animals which had served as controls for the challenging inoculations in Experiments II and III had also become resistant to challenge as judged both by blood smears and subinoculation. However, the three normal control animals became infected and were positive on subinoculation for periods of 4 to 9 weeks. The animals of Experiment II were challenged for the third time 14 months after immunization along with two untreated control animals. This challenge, unlike those given 1 week and 8 months after immunization, produced detectable infections both in immunized and untreated control cattle. However, the prepatent periods of the immunized animals were prolonged (7 days) as compared to those of their controls (3 and 4 days). Previously infected animals which had undergone spontaneous cures again resisted the challenging infection.

Experiment IV

To determine whether or not resistance to a different strain of T. rhodesiense was elicited by immunization or infection of cattle with the Wellcome strain, the animals of Experiment III were rechallenged with a more recently isolated strain of T. rhodesiense (EATRO #1886). No obvious differences in prepatent periods or intensity of infections were observed between immunized, spontaneously cured, or untreated control animals.

Inoculations of irradiated T. rhodesiense induced a strong resistance of a lasting nature in cattle. Immunized animals were considered to be parasite-free as a result of negative blood smears and repeated subinoculations of blood into mice. These results confirm and extend those reported by Duxbury and Sadun in rats and mice. On the basis of our findings one cannot determine with certainty whether the immunity observed in cattle to T. rhodesiense is indeed a sterile immunity. The possibility exists that the predilection of the parasite for connective tissues as reported in other hosts might have resulted in negative subinoculations. This seems unlikely, however, in view of the fact that all control animals were positive upon subinoculation while immunized animals were consistently negative. Blood samples of some control animals were not positive on every subinoculation. Whether the number of parasites in the subinoculated blood was below the infectivity level for mice or whether the parasites were sequestered in the tissues on these occasions is not known.

When cattle were immunized by infection and chemotherapy, a resistance against challenge developed which persisted for 8 months. Our results are in agreement with these observations since immunity persisted at a high level for at least 8 months in the immunized cattle.

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Table 2

Challenge with Homologous Strain of T. Rhodesiense
1 Week After Immunization

Exp. II

Group	Weeks After Challenge 0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Immunized																	
6359	-	-	-	-		-		-	-		-		-				
6360	-	-	-	-		-		-	-		-		-				
Control																	
6346	-	⊕	⊕	+		⊕	0	+	+		-						
6364	-	⊕	⊕	⊕		+		+	+		-						

Exp. III

Immunized																	
6340	-		-	-		-		-		-					-		-
6351	-		-	-		-		-		-					-		-
6358	-		-	-		-		-		-					-		-
Control																	
6350	-	0	⊕	⊕		+		+		+					-		-
6374	-	0	⊕	+		-		+		+					-		-
6390	-	0	⊕	+		+		+		+					-		-

- Negative subinoculation; + positive subinoculation
0 Weeks during which parasites were observed microscopically

Although some degree of immunity was detected in these animals 14 months after immunization it was not complete. The animals which had undergone infections resulting in spontaneous cure were resistant to reinfection both at 8 and 14 month intervals after infection, indicating that the stimulus provided by the actual infection induces a more persistent immunity than that produced by irradiated parasites.

The immunity demonstrated in our experiments is surprising in view of the well known ability of trypanosomes to vary their antigenic constituents. We considered the possibility that our monomorphic laboratory strain (Wellcome) might have lost its ability to undergo antigenic variation after repeated blood passage in laboratory animals. However, antigenic variations were found to occur with this strain. Moreover, results from experiments involving mice and rhesus monkeys indicate that a strong resistance could also be induced in these animals by immunization with irradiated trypanosomes of a recently isolated strain of T. rhodesiense.

Immunization with attenuated parasites has generally been more effective than that achieved by immunizing with non-living trypanosomal antigens. Although the irradiated trypanosomes are apparently not capable of replication, they remain motile for some time and presumably retain a portion of their metabolic integrity. Strain specific protection induced by exoantigens has been reported and it is likely that release of metabolic antigens by the attenuated but living parasites is responsible for the strong immunity induced by irradiated trypanosomes.

It is possible that rat host contaminants of the immunizing inocula played a role in the resistance observed but this seems unlikely in view of the fact that the contaminants were maximal in inocula which were less protective (Experiment I) and minimal in inocula which were more protective (Experiments II and III). Resistance was also not present in either immunized or recovered animals when challenged by a different strain.

Table 3
Development of Immunity in Rats after a Single Inoculation of Irradiated Trypanosoma rhodesiense

Group	Trypanosomes	Challenge (Days after Immunization)	Survivors Challenged	Day of Death
1	1.6×10^8	22	2/3	14
2	3.9×10^8	17	2/3	20
3	2.1×10^8	15	3/3	-
4	2.0×10^8	11	2/3	14
5	2.5×10^8	9	1/2	13
6	2.2×10^8	7	3/3	-
7	controls	-	0/10	5.6

Although some degree of immunity was detected in these animals 14 months after immunization it was not complete. The animals which had undergone infections resulting in spontaneous cure were resistant to reinfection both at 8 and 14 month intervals after infection, indicating that the stimulus provided by the actual infection induces a more persistent immunity than that produced by irradiated parasites.

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2. Parasitological, clinical, hematological, serological and pathological observations in rhesus monkeys infected with Trypanosoma rhodesiense.

Fifteen young adult rhesus monkeys (Macaca mulatta) of both sexes weighing 4 to 6 kg at the beginning of the experiments were used. Six of these were immunized with irradiated trypanosomes and challenged later, and 9 were challenged without previous immunization. Additional animals were kept as uninfected controls. No differences in any of the parameters studied were observed between the immunized monkeys and the uninfected controls. Therefore, in the course of these studies a comparison was made between the immunized monkeys, which remained healthy after they received challenging inoculations, and the unimmunized animals, which invariably developed fatal infections after challenge.

The strain of T. rhodesiense (EATRO 1886) was isolated from a human patient in Uganda and passaged once in a rat before it was frozen and

shipped to our laboratory. Monkeys and rats were infected from this suspension. Trypanosomes for immunization and challenge were collected from heavily parasitized donor rats. In some of the experiments the parasites were counted with a hemacytometer and inoculated in whole blood; in others, the trypanosomes were separated from rat blood in a DEAE cellulose column with a phosphate-buffered saline-glucose (PS) solution. The parasites recovered from the column were washed twice by centrifugation in PSG solution and their numbers determined by electronic particle counts.

For immunization the parasites were exposed to a 60 kr dose in a Gammacell 220 cobalt-60 irradiator. The immunizing and challenge doses were inoculated intravenously. During the immunization period and after challenge wet blood smears were examined microscopically for trypanosomes. Susceptible mice were subinoculated with 0.2-0.4 ml of blood from each experimental animal in an effort to detect trypanosomes not seen on direct examination. These mice were examined for at least 30 days before they were considered to be free of infection. The monkeys were observed for 90 days after challenge unless they died earlier. The numbers of parasites used for inoculation and challenge as well as the immunization schedules have been described elsewhere.

Blood was collected from each monkey at regular intervals to determine hematocrit, hemoglobin levels and erythrocyte, reticulocyte, leucocyte, platelet and differential counts. Serum specimens were also obtained before exposure and at weekly intervals thereafter for biochemical determinations included glucose, blood-urea nitrogen, serum glutamic oxalacetic and glutamic pyruvic transaminase (SGOT and SGPT), bilirubin, creatinine, total protein and serum electrophoresis.

Serological determinations included fluorescent antibody tests conducted according to the method described earlier except that 0.001% Evans blue was used as a counterstain instead of the rhodamine bovine albumin described in the previous method.

At necropsy all the major organs were observed, removed and weighed and representative portions of them were fixed in 10% buffered formalin. The fixed tissues were embedded in paraffin, mounted in paraffin blocks and sections 5-6μ thick were cut. The tissues were stained with hematoxylin and eosin. For detailed studies of the kidneys of infected animals, sections were stained with periodic acid Schiff (PAS) and methanamine silver stain. In addition 1 mm cubes of formalin fixed renal tissue were post-fixed in osmium, dehydrated, embedded in Epon epoxy resin and sectioned at approximately 100 mμ with a diamond knife. The sections were doubly stained with uranyl acetate and lead citrate and examined with an electron microscope (RCA EMU -3G).

A. Parasitological observations

No trypanosomes were found in the blood of the monkeys before experimental infection and for several days afterward. Patency, determined by examining thin blood smears, varied from 4 to 8 days in unimmunized

monkeys inoculated with approximately 10^6 trypanosomes. There was great fluctuation from day to day in the numbers of trypanosomes present in the peripheral blood of these animals. Hemacytometer counts revealed as many as 700 million parasites per milliliter of blood in some of the monkeys, then less than 2 million per milliliter a few days later. Maximum parasitemias were usually attained after 19-25 days and survival time varied from 6-49 days. The monkeys tended to die earlier as the infection was passaged, though the differences observed in time of death during the first 3 passages from the original inoculum were not significant.

B. Clinical observations

All immunized and subsequently challenged animals gained weight in the course of the experiment. Conversely, all nonimmune animals began losing weight early after exposure and continued to do so until death.

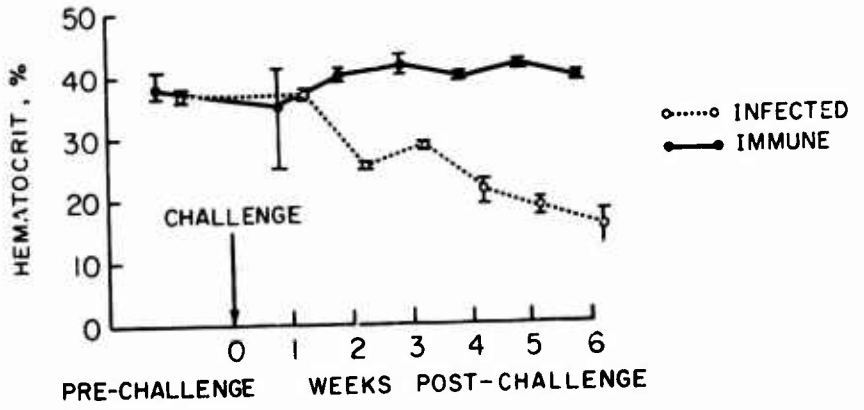


Fig. 1 Packed cell volumes in rhesus monkeys infected with Trypanosoma rhodesiense.

Whereas the immunized animals gained an average of 450 gm during the course of the experiment, the unimmunized infected animals lost on the average nearly 1 kilogram during the 6 weeks following challenge. No other obvious signs of illness were observed during the first few weeks after inoculation. However, the nonimmune animals showed progressive signs of inactivity and their mucosae became increasingly pale.

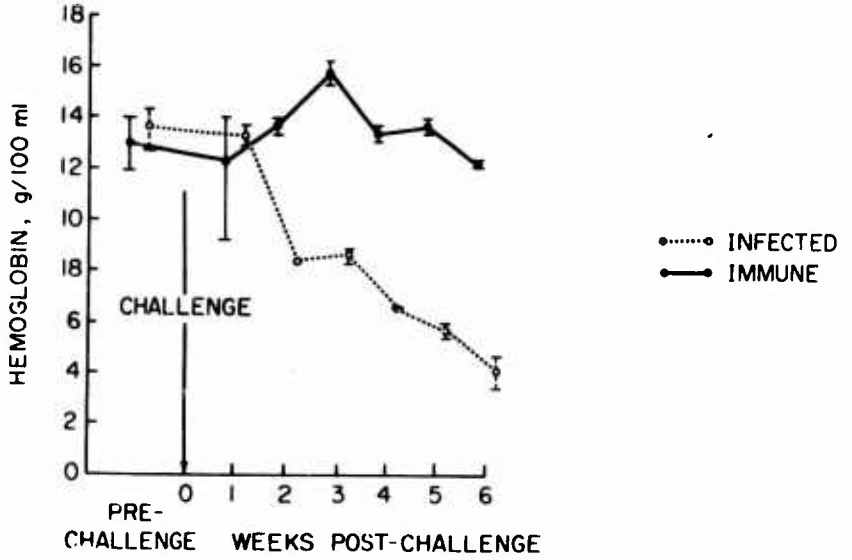


Fig. 2 Hemoglobin in rhesus monkeys infected with Trypanosoma rhodesiense.

C. Hematological studies

As shown in Figs. 1-4, there was progressive drop in the per cent hematocrit, hemoglobin, and erythrocyte and platelet counts in the infected animals. Concomitantly, there was an increase in reticulocyte count (Fig. 5). No consistent significant changes were observed in the total and differential leucocyte determinations.

D. Biochemical observations

No significant alterations in serum bilirubin and glucose were observed in the infected animals. A marked increase in blood urea nitrogen (BUN) was observed only in one monkey late in the infection. This reached a level of 50 mg per cent as compared to an average of 16 mg per cent in the immunized uninfected controls (Fig. 6). There was a marked increase in transaminases (SGOT and SGPT) in the infected monkeys beginning early after challenge and reaching levels of 280 and 214 units respectively (Figs. 7, 8). Similarly there was an increase in creatinine (Fig. 9) in the infected animals. Total serum proteins (Fig. 10) decreased as the infection progressed. This was due primarily to a striking decrease in albumin (Fig. 11) which was not offset by a moderate increase in gamma globulin (Fig. 12). Alpha 1, alpha 2 and beta globulin levels in the infected animals remained essentially unaltered.

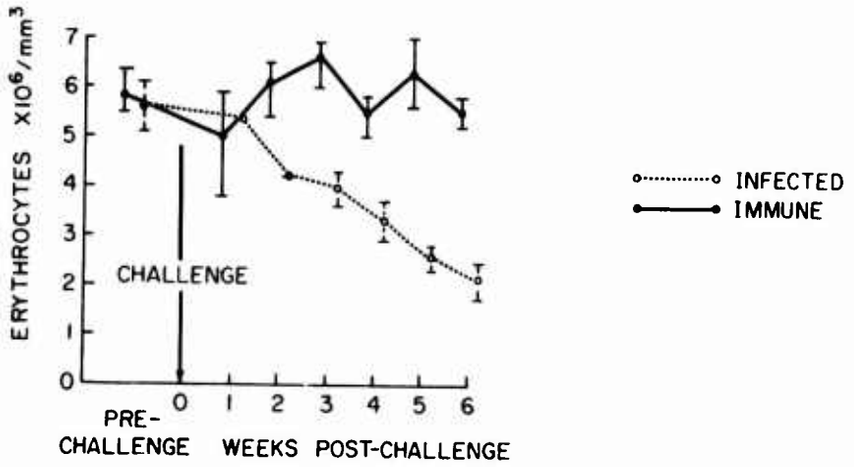


Fig. 3 Erythrocytes in rhesus monkeys infected with Trypanosoma rhodesiense.

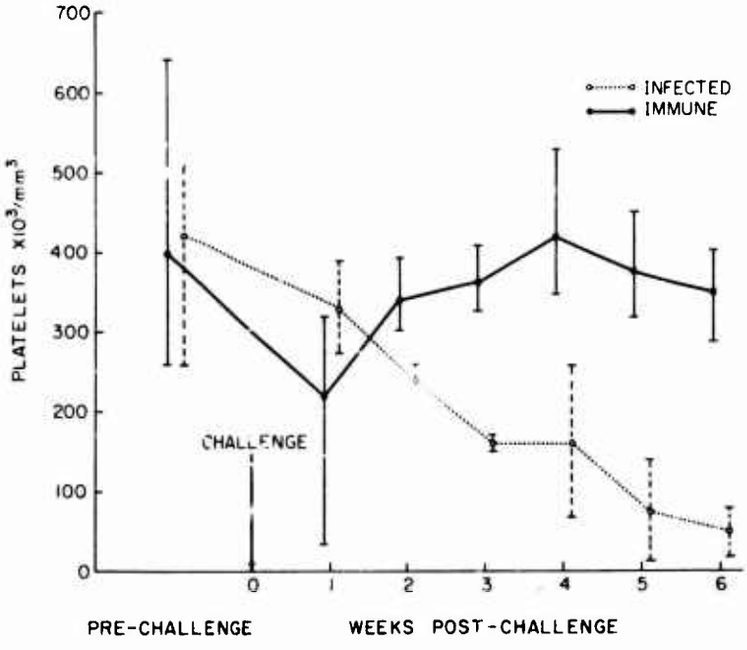


Fig. 4 Platelets in rhesus monkeys infected with Trypanosoma rhodesiense.

E. Serological results

Fluorescent antibody tests in the immunized animals indicated a conversion from negative to positive within the first two weeks after immunization was begun. The maximum titer, 1:64, was obtained three weeks after the first immunizing dose. Challenge failed to produce an anamnestic response and the titers gradually decreased until all animals reverted to negative 19 weeks after the first immunization (13 weeks after challenge). Conversely, the unimmunized controls became positive during the second week after challenge and maintained titers of 1:128 or higher until death. Titers as high as 1:512 were obtained as early as 5 weeks after challenge.

F. Pathological observations

The infection was usually fatal within two months. The gross pathological changes varied in degree and distribution and included weight loss, anemia, subcutaneous edematous thickening of the ventral abdominal and scrotal walls, increased fluids in the body cavities (especially of the abdominal cavity), mild hydropericardium, splenomegaly, slight hepatomegaly, mild lymphadenopathy and congested lungs. Microscopically, changes were consistently observed in the liver, heart, kidney, spleen and lymph nodes. In the liver, perivascular mononuclear cell cuffs involving portal triads and central veins and an increased occurrence of large mononuclear cells in dilated sinusoids were seen (Fig. 13). Red blood cells and many trypanosomes were observed in

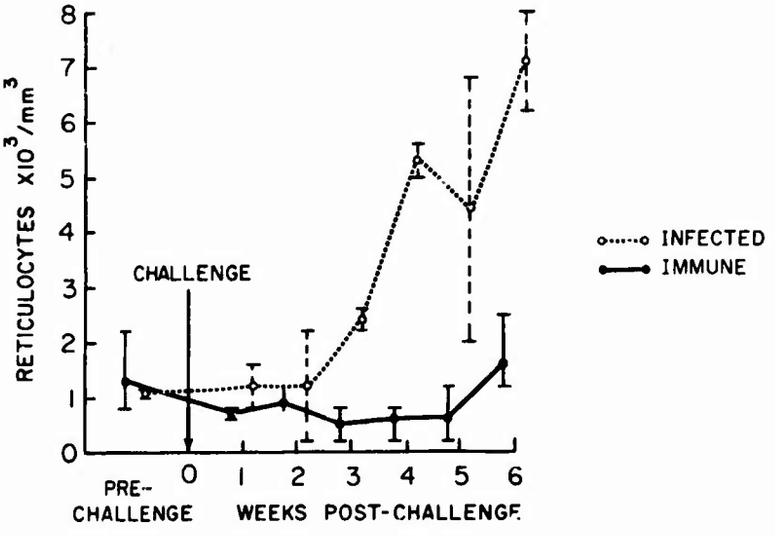


Fig. 5 Reticulocytes in rhesus monkeys infected with Trypanosoma rhodesiense.

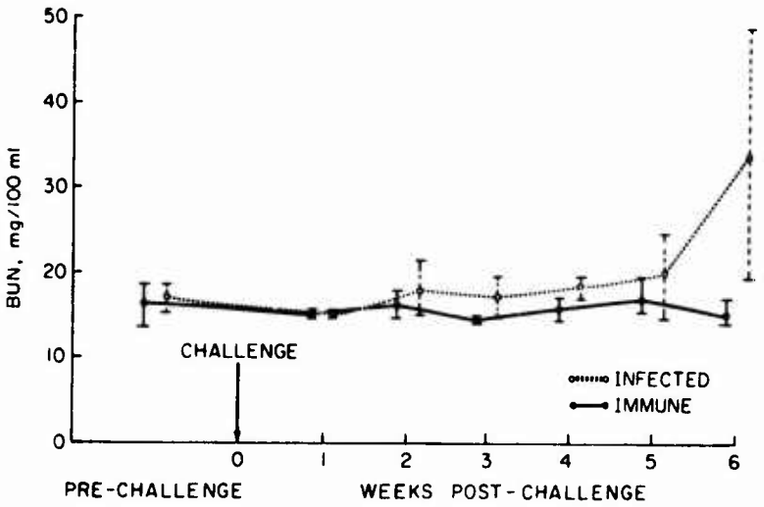


Fig. 6 Blood urea nitrogen in rhesus monkeys infected with Trypanosoma rhodesiense.

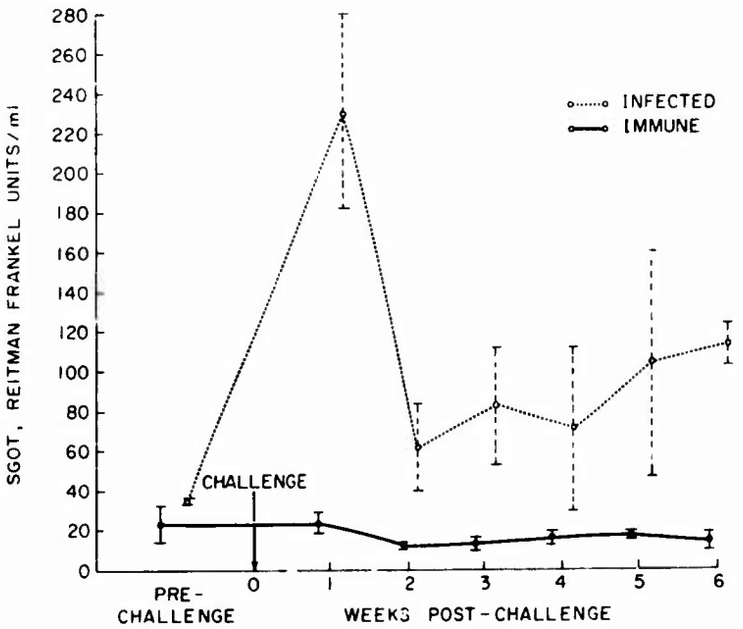


Fig. 7 Serum glutamic oxalacetic transaminase in rhesus monkeys infected with Trypanosoma rhodesiense.

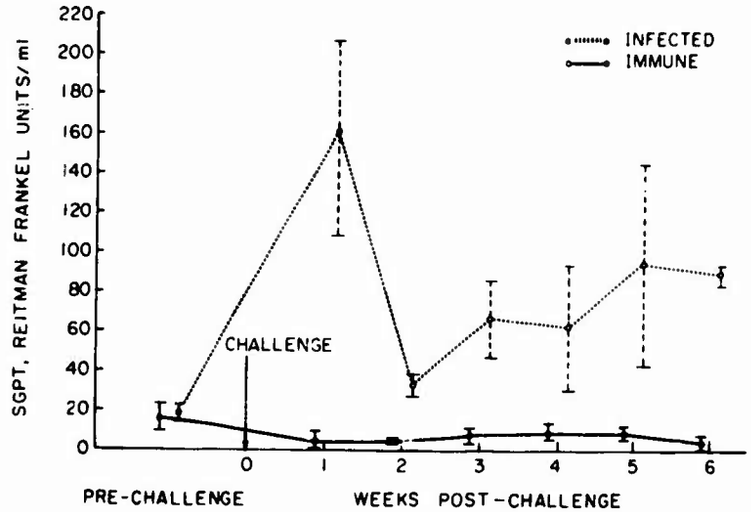


Fig. 8 Serum glutamic pyruvic transaminase in rhesus monkeys infected with Trypanosoma rhodesiense.

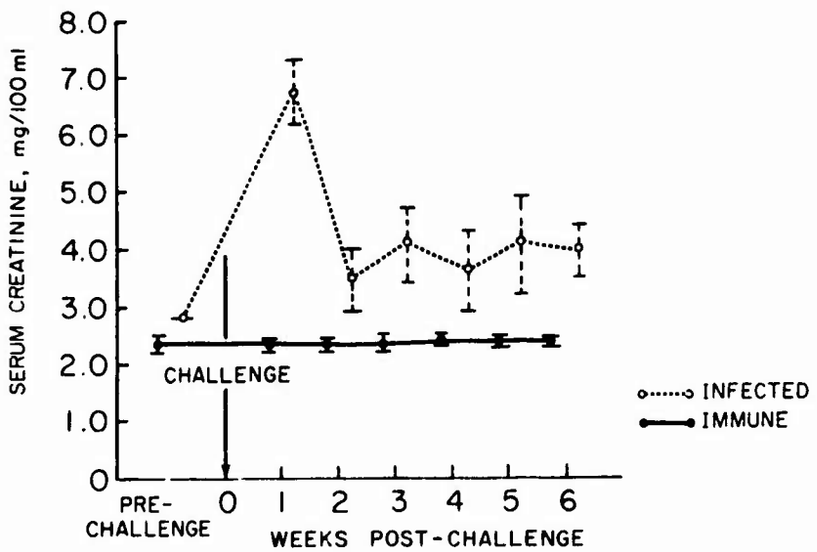


Fig. 9 Serum creatinine in rhesus monkeys infected with Trypanosoma rhodesiense.

some of the mononuclear cell cuffs (Fig. 14). Histopathological observations of the heart muscle showed an increase of mononuclear cells in intramuscular capillaries and the adjacent intramuscular connective tissue (Fig. 15). This change was frequently observed in subepicardial capillaries and the surrounding stroma. Focal hemorrhages and trypanosomes accompanied the mononuclear cells in some areas. In a few cases changes in myocardial fibers were observed indicating myocarditis.

In the spleen and lymph nodes there was lymphoid and reticuloendothelial hyperplasia, focal hemorrhages and congestion. Erythrophagocytosis was commonly observed in these organs. The lungs were congested and contained a mild infiltration of mononuclear cells in septal spaces. In one monkey a mild to moderate nonsuppurative encephalitis was evident. Large mononuclear cells were located around vessels adjacent to the ventricle. Swollen glia cells were also recognized. Trypanosomes were frequently observed within the lumen and the perivascular adventitia of vessels in many organs.

In three of the infected monkeys there was evidence of proliferative glomerulonephritis. The glomeruli were diffusely involved with a process characterized by hypercellularity of the glomerular tuft, exudation, and sclerosis of the mesangium. The capillary lumens were largely obliterated and the capillary loops appeared to have collapsed (Fig. 16). The PAS and methanamine silver stains revealed thickening and duplication of the capillary basement membrane (Fig. 16). Electronmicroscopic analysis of formalin fixed material from one monkey confirmed the thickening and duplication of the basement membranes. There was swelling and simplification of the visceral epithelial cells with apparent focal loss of filtration slits. Immune complexes were not seen. Some glomeruli revealed focal synechiae and a few showed crescents (Fig. 16). The proximal tubules showed increased numbers of PAS positive cystosegrosomes. Occasional tubules contained proteinaceous casts, some of which included polymorphonuclear leucocytes (Fig. 16). Proteinaceous casts were also seen within the collecting ducts. Within the interstitium there were focal infiltrates of mononuclear cells, particularly in the perivascular regions. The vessels were unremarkable.

These studies in general indicate that the rhesus monkey is a valuable model for investigating the natural history of Rhodesian trypanosomiasis and some of the pathogenic mechanisms operating in this disease. No abnormalities attributable to inoculation with virulent organisms of T. rhodesiense were observed in any of the immunized monkeys, but in all of the unimmunized animals parasites were readily detected in the peripheral blood after a brief prepatent period. At autopsy the salient features of infection with this parasite were reticuloendothelial hyperplasia of the spleen, liver and lymph nodes and a perivascular mononuclear cell infiltrate in the liver, kidneys and occasionally the heart. A proliferative glomerulonephritis was also seen in the kidneys of three animals. Although fluorescent antibody reactions were obtained with the serum of all animals following exposure to infection, the titers in the unimmunized monkeys were con-

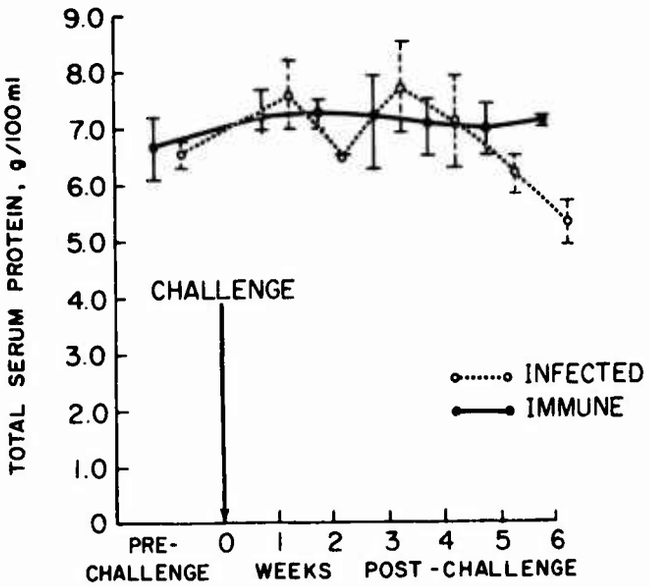


Fig. 10 Total serum protein in rhesus monkeys infected with Trypanosoma rhodesiense.

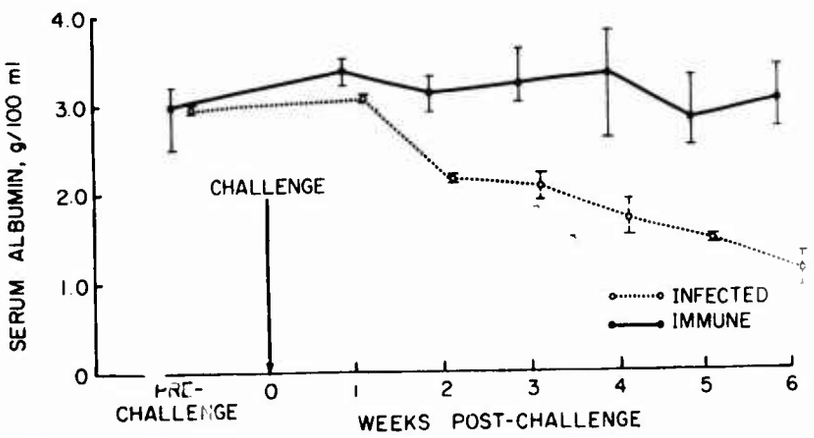


Fig. 11 Serum albumin in rhesus monkeys infected with Trypanosoma rhodesiense.

siderably greater than in those of the immunized andprotected monkeys. This suggest that the antibodies detected are not protective in nature. Serum biochemical changes were recorded in the infected animals. These included increases in transaminases, creatinine, blood urea nitrogen and gamma globulin. There was a decrease in total protein which could be accounted for almost entirely by marked decrease in albumin. Increased serum catabolism, decreased albumin synthesis or abnormal loss

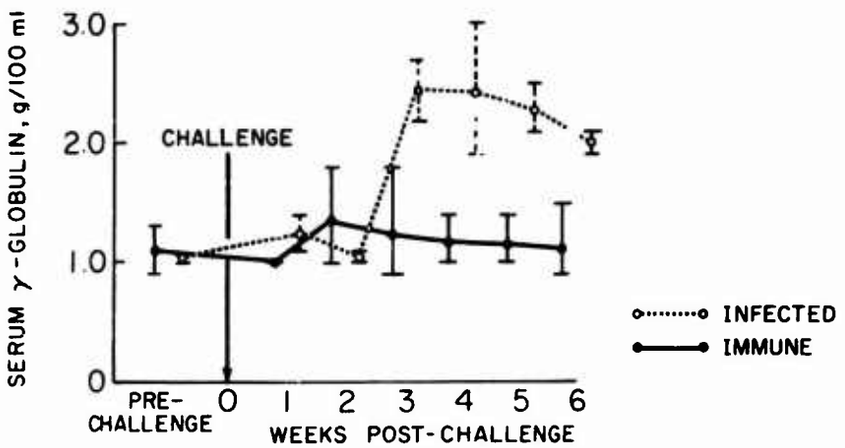


Fig. 12 Serum gamma globulin in rhesus monkeys infected with Trypanosoma rhodesiense.

in the urine may be involved. Moreover, considering the high parasitemias recorded, one would expect that large amounts of globulin would be absorbed in antigen-antibody complexes. Most of the transaminase activity probably arises from the parasite itself. The extent to which damaged host tissues contribute is not clear. Although it has been reported that trypanosomiasis produces hypoglycemia we found no evidence to support this observation.

In general, the most rapid and remarkable changes observed were in the hematological parameters. The total red blood cell count and the hematocrit values dropped rapidly within two weeks after infection and continued to decrease until death. Similarly the hemoglobin levels were considerably reduced, in one animal to one-sixth of its earlier level in six weeks. The number of platelets also dropped dramatically. Corresponding with these changes there was a rapid increase in reticulocytes.

The present studies provide strong evidence of the occurrence of disseminated intravascular coagulation in association with T. rhodesiense

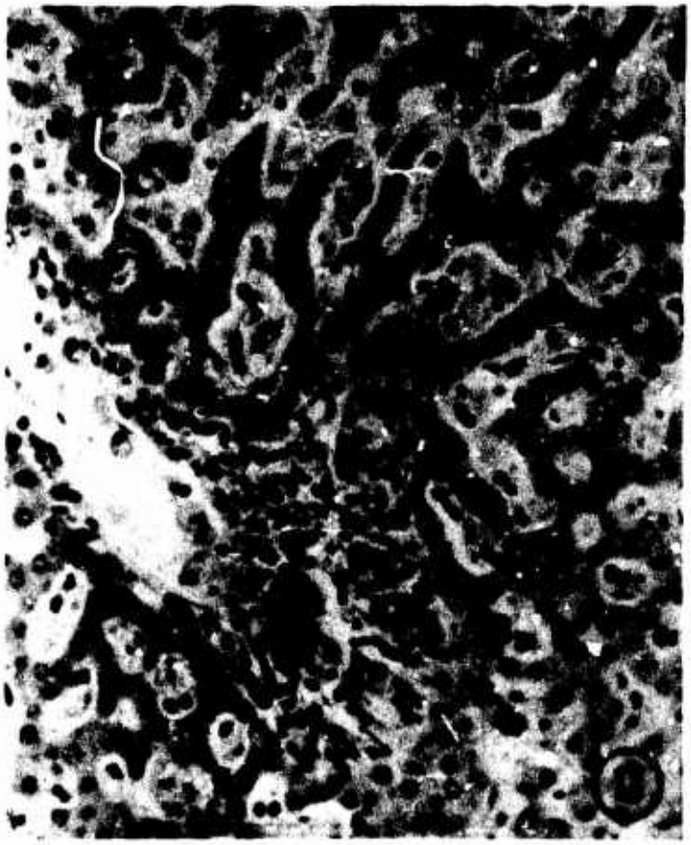


Fig. 13 Section of liver showing mononuclear cell infiltrate in portal triad. Note dilated sinusoids containing mononuclear cells and lined by swollen Kupffer cells. H&E x 200

infection in rhesus monkeys. Studies are now underway to determine the possible mechanisms of it. However, it is unlikely that liver functional impairment is responsible for the coagulation abnormalities observed in these experimental animals since microscopic examination of livers from the infected monkeys revealed no evidence of cirrhosis and only minimal hepatic parenchymal abnormalities. The occurrence of disseminated intravascular coagulation provides one possible explanation for the development of thrombocytopenia in these animals. Also, one cannot exclude immunologically mediated phenomena as another possible cause of the thrombocytopenia. Antigen-antibody complexes have been recognized as mediators of both platelet destruction per se and disseminated intravascular coagulation. Therefore this mechanisms should be given consideration n view of the proliferative glomerulonephritis which was observed in the kidneys of three animals. The interrelationship of these processes and the role, if any, that antigen-antibody complexes play in the thrombocytopenia, in the anemia, in the glomerulonephritis and in the levels of globulin and complement require further studies.



Fig. 14 Perivascular infiltrate surrounding hepatic vein. Arrow indicates extravascular trypanosome. H&E x 820



Fig. 15 Section of right atrium showing perivascular infiltrate of mononuclear cells surrounding the capillaries of the myocardium. H&E x 200



Fig. 16 Section of kidney revealing three glomeruli. Note increased sclerosis of mesangium, obliteration of capillary lumina, and duplication of basement membranes. Arrow indicates a crescent. A proteinaceous cast containing white cells is seen at the upper left. PAS x 200

3. Immunization of monkeys against a rec[illegible]y isolated human strain of Trypansoma rhodesiense by use of gamma i[illegible]tion.

Previous investigations have demons[illegible]d that immunity can be induced against African trypanosomiasis in r[illegible]nts and cattle by inoculating them with irradiated trypanosomes. Since the studies with T. rhodesiense utilized the laboratory-adapted Wellcome strain, further investigations were conducted to determine whether the same degree of protection could be obtained with a recently isolated human strain, EATRO No. 1886. In addition, the investigations were extended to include subhuman primates.

2 experiments involving 11 rhesus monkeys were conducted (Table). The unimmunized controls in both experiments died as a result of the infection 12-49 days after challenge. In contrast, 5 of the 6 immunized animals survived challenge and remained parasite-free throughout a 90-day observation period. Clinical, haematological and pathological findings in the immunized and challenged monkeys, with the single exception

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of No. 611 which became infected, were similar to those of normal monkeys. Conversely, the unimmunized and challenged monkeys developed progressive anaemia, thrombocytopaenia, reticulocytosis and signs of progressive severe illness.

Table 4

Development of immunity in rhesus monkeys inoculated with irradiated (60 kr.) *Trypanosoma rhodesiense* (LATRO No. 1886) and challenged 3 weeks later with 1×10^4 unirradiated trypanosomes.

Immunizing inoculations					
Exp. No.	Monkey No.	No. Doses	No. trypanosomes	Prepatent period (days)	Survival time (days)
1	628	0	0	5·0	44
	723	0	0	5·0	31
	611	6	1×10^8 to $1{\cdot}8 \times 10^9$	13·0	75
	664	6	1×10^8 to $1{\cdot}8 \times 10^9$	*	>90
	670	6	1×10^8 to $1{\cdot}8 \times 10^9$	*	>90
2	403	0	0	8·0	12
	484	0	0	6·0	47
	505	0	0	5·0	49
	506	6	2×10^8 to $1{\cdot}3 \times 10^9$	*	>90
	523	6	2×10^8 to $1{\cdot}3 \times 10^9$	*	>90
	833	6	2×10^8 to $1{\cdot}3 \times 10^9$	*	>90

*No parasites were recovered from the blood at any time after challenge.

4. Clinical observations on cattle experimentally infected with Trypanosoma congolense.

The extent and severity of the problems caused by trypanosomiasis of domestic animals in Africa have been well known for many years and various aspects of the disease have received considerable attention by researchers. Although many pathological changes occuring in host animals have been attributed to trypanosomiasis, in some instances the findings appear to be of a fragmentary and inconclusive nature. This may be due partially to differences in virulence of separate species of trypanosomes and of strains within one species, as well as by host factors such as age, condition, sex, breed and the presence of concomitant infections.

In the course of experimental immunization studies, we had an opportunity to document the course of disease in a group of cattle infected with Trypanosoma congolense under similar conditions. Hematologic, parasitologic, serum chemical and serologic procedures were conducted at regular intervals following infection and histopathological observations were made at death.

Experimental Animals

Two Hereford steers and nine nonpregnant Hereford heifers, weighing from 505 to 845 pounds, were selected from the Veterinary Department herd at Kabete, Kenya, and used in these studies. The age of the animals ranged from 1.3 to 2.8 years. The cattle were stabled at the Trypanosomiasis Research Laboratory in Kabete, Kenya; and they grazed in a nearby pasture during the day. All animals were weighed and dipped in an acaracide weekly.

Parasites

The Trans Mara I strain of T. congolense which was isolated from an infected cow in the Trans Mara area, near the Kenya-Tanzania border, in 1966 was used throughout the experiment. The strain has been stored as a stabilate in dry ice and maintained in cattle by blood passage. For inoculation, trypanosome concentrations were calculated from counts of the numbers of trypanosomes per 10,000 erythrocytes (RBC's) on thin blood films and total RBC counts per mm. Blood for inoculations was diluted with phosphate buffered saline with 5% glucose added and injected into the jugular vein. Parasite concentrations on individual animals were determined daily by counting the number of trypanosomes in 50 oil immersion fields containing approximately 200 RBC's per field in thin blood smears; if no parasites were seen at this level, 200 oil immersion fields on thick blood smears were observed.

Collection of Specimens

Thick and thin blood smears were usually obtained 6 days per week at approximately 9 A.M. by puncturing the tip of the tail. Blood for biochemical, hematological, and serological determinations were collected (from the jugular vein) in tubes, allowed to clot overnight at 4°C and stored at -20°C until assayed. Animals found dead or moribund were transported to the Diagnostics Section of the Veterinary Research Laboratory for post mortem examinations. Impression smears were made from the cut surfaces of organs of recently killed animals. Tissue specimens were collected in buffered formalin for histopathologic evaluation.

Hematology

Erythrocytes and leukocytes were counted in a Neubauer Chamber using Hayem's solution as diluent for erythrocytes and 1% acetic acid solution for leukocytes. The microhematocrit method was used to determine packed cell volumes (PCV). Thin blood smears from each animal were fixed in methanol before staining with Giemsa's stain. Differential white cell counts were done by classifying 100 leukocytes on thin smears.

Serum Chemistry

Serum glutamic oxaloacetic transaminase (SGOT), glucose, creatinine and total protein levels were determined by the methods described by Williams et al. Levels of urea nitrogen were determined by a modification of the urease method. Total serum bilirubin values were obtained using a modification of the method described by Jendrassik and Grof. Serum electrophoresis on cellulose acetate strips was performed by the Beckman Microzone technique.

Immunization

An attempt was made immunize 3 animals with irradiated trypanosomes. Blood (60 ml) was obtained from the donor cattle during the first peak of parasitemia. Tubes containing the blood and heparin were put on ice and transported to the Veterinary Faculty, Kabete where they were irradiated in a Co^{60} source at a level of approximately 60 Kr. The dose rate at the time of the experiment was 3.3 Kr per minute. The tubes were placed in a plastic rack inside the irradiator and were not cooled during irradiation. After the desire level of radiation had been obtained the tubes were again placed on ice and taken back to the Trypanosomiasis Research Laboratory where 20 ml of irradiated parasitized blood was injected intravenously into each of the 3 experimental animals. Four injections of irradiated parasites were given at about weekly intervals and the number of trypanosome per injection ranged from 1×10^8 to 8.0×10^8. A week after the fourth immunization the 3 immunized as well as 3 control animals were challenged intravenously with 2×10^5 viable T. congolense.

Clinical Observations

The animals in these experiments were separated into 3 groups. Group I consisted of 5 animals which were injected with viable T. congolense organisms and used as donors of trypanosomes for immunization and challenge. Group II contained 3 animals which served as controls for the immunized cattle which were designated as Group III. The only observable protective effect elicited by the irradiated parasites in the animals in Groups III when compared to their controls in Group II was an increase of 2 days in the length of their prepatent periods and a corresponding delay in the development of fever. After the onset of parasitemia the course of infection in 2 immunized animals was similar to that of the controls. The other immunized animal died relatively soon after challenge but its death was complicated by concomitant anaplasmosis. Since the eight cattle in Groups I and II received no pretreatment, the course of infection in these animals were studied closely.

The length of the prepatent periods, but not the duration of the disease, was related to the numbers of trypanosomes injected. After the onset of patency, trypanosomes were detected daily for the first seven weeks in all 8 animals. After the seventh week of infection,